Molecular mechanisms of porcine circovirus 2 replication and pathogenesis

By

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Abstract

The non-pathogenic porcine circovirus type 1 (PCV1) was originally isolated as a persistent contaminant of the porcine kidney cell line PK-15. Whereas, porcine circovirus type 2 (PCV2) causes postweaning multisystemic wasting syndrome (PMWS) in pigs, which is devastating to the swine industry. My objectives were to determine the effect of maternally derived antibodies on PCV2 infection, assess the role of 2 amino acid substitutions in the PCV2 capsid protein in PCV2 attenuation, evaluate the effect of Rep gene exchange between PCV1 and PCV2 on growth characteristics of a chimeric PCV2, and evaluate the role of open reading frame (ORF) 3 of PCV2 in virus replication and pathogenesis in pigs.

Under field conditions, PCV2 infection is widespread and most breeding pigs are seropositive. Assessment of the role of PCV2 maternal antibodies in preventing PCV2 infection in piglets provided evidence that higher levels of maternal antibody provide more protection to piglets.

Two amino acid substitutions in the PCV2 capsid protein that enhanced virus replication *in vitro* and attenuated the virus *in vivo* were evaluated for their pathogenicity in pigs. The results indicated that P110A and R191S are collectively responsible for virus attenuation.

PCV1 replicates better in PK-15 cells and grows at least 1-log titer higher than PCV2. A chimeric PCV with the rep gene of PCV1 replacing that of PCV2 in the genomic backbone of PCV2 replicated more rapidly than PCV1 and PCV2, and more efficiently than PCV2, although to a titer similar to PCV1.

The ORF3 of PCV2 is believed to encode a protein involved in apoptosis. The ORF3 start codon was mutated from ATG to GTG and the resulting mutant muPCV2 was infectious *in vitro* and in pigs; therefore ORF3 is dispensable for virus replication.

The pathogenicity of muPCV2 was compared with PCV2 *in vivo*. Delayed viremia and seroconversion, decreased viral loads, lower level of IgG antibodies, and lower amounts of PCV2 antigen in mesenteric lymph nodes suggested attenuation of muPCV2. However, there was no significant difference in histological or gross lesions in tissues between PCV2- and muPCV2-inoculated groups. The role of ORF3 in attenuation needs to be further elucidated.

Dedication

I dedicate my dissertation to my husband and to my family with love and appreciation.

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Attributions

Chapter 2 of this dissertation was carried out by N.E. McKeown, T. Opriessnig, P. Thomas, D.K. Guenette, F. Elvinger, M. Fenaux, P.G. Halbur, and X.J. Meng.

N.E. McKeown- generated virus stock, stock titration, sample processing, Q-PCR
T. Opriessnig- pig inoculation, sample collection
P. Thomas- pig care
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F. Elvinger- statistical analysis
M. Fenaux- generated infectious DNA clone
P.G. Halbur- experimental guidance
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Chapter 3 of this dissertation was carried out by N.E. McKeown, T. Opriessnig, F. Elvinger, M. Fenaux, P.G. Halbur, and X.J. Meng.

N.E. McKeown- constructed mutants, prepared virus stock, Q-PCR, sequencing
T. Opriessnig- pig inoculation, evaluation, sample collection, histopathology, immunohistochemistry
M. Fenaux- discovered mutants
F. Elvinger- statistical analysis
P.G. Halbur- experimental guidance
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Chapter 4 of this dissertation was carried out by N.M. Juhan and X.J. Meng.

N.M. Juhan- all experimental work
X.J. Meng- experimental guidance

Chapter 5 of this dissertation was carried out by N.M. Juhan and X.J. Meng.

N.M. Juhan- all experimental work
X.J. Meng- experimental guidance

Chapter 6 of this dissertation was carried out by N.M. Juhan, T. LeRoith, T. Opriessnig, and X.J. Meng.

N.M. Juhan- constructed mutant, pig inoculation, sample collection and processing, Q-PCR, sequencing, statistical analysis
T. LeRoith- necropsy, gross pathology, histopathology
T. Opriessnig- immunohistochemistry
X.J. Meng- experimental guidance

Chapter 8 of this dissertation was carried out by N.E. McKeown, M. Fenaux, P.G. Halbur, and X.J. Meng.

N.E. McKeown- all experimental work, phylogenetic analysis
M. Fenaux- experimental guidance
P.G. Halbur- experimental guidance
X.J. Meng- experimental guidance

Table of Contents

List of Figures

List of Tables

General Introduction

Porcine circovirus type 1 (PCV1) was discovered in 1974 as a persistent contaminant of the porcine kidney PK-15 cell line (98). It has since been shown that PCV1 is a ubiquitous swine virus that does not cause any disease (6, 24, 25, 96). In 1991, a new pig disease, called postweaning multisystemic wasting syndrome (PMWS), was recognized first in Canada, and porcine circovirus type 2 (PCV2) was found to be the primary causative agent (7, 60). The symptoms of clinical PMWS include severe progressive weight loss, dyspnea, lymph node enlargement, diarrhea, pallor, and jaundice in pigs of 7-15 weeks of age (35, 60). PMWS associated morbidity ranges from 4-30% on affected farms and approximately 70-80% of those pigs die (84). The mechanisms of PCV2 pathogenesis in PMWS are not well understood.

The virions of PCV1 and PCV2 are nonenveloped and icosahedral (95), and their genomes contain single-stranded circular DNA of 1759 nt and 1768 nt, respectively (55, 95, 98). The genomic organization of PCV1 and PCV2 are similar: each has 2 main open reading frames (ORF). ORF1 encodes the replication protein (Rep) (54) and ORF2 encodes the major capsid protein (62). Recently a 3rd ORF, ORF3, was identified for PCV2, but was not found in PCV1 (47). ORF3 was found to induce apoptosis *in vitro* (47) and was involved in pathogenesis in mice (46).

The objectives of this dissertation are to (1) determine the effect of maternal antibodies on PCV2 infection; (2) evaluate the role of 2 amino acid substitutions on the capsid protein in PCV2 attenuation; (3) assess the replication kinetics of a chimeric PCV2 containing the Rep gene of PCV1; and (4) evaluate the role of ORF3 in PCV2 replication and pathogenesis in pigs. Completion of these objectives will further the understanding of PCV2 biology and pathogenesis.

Chapter 1
Literature Review

1.1 History

Porcine circovirus type 1 (PCV1) was first described in 1974 as a persistent contaminant of the PK-15 cell line ATCC CCL-33 (98). Since its identification, PCV1 has been demonstrated to be a ubiquitous swine virus that does not cause any disease in pigs (6, 24, 25, 96). In 1991, a variant strain of PCV was identified in pigs with postweaning multisystemic wasting syndrome (PMWS) in Canada (7, 60). This variant strain was deemed PCV2 and has since been identified from pigs throughout the world (48, 74) in connection with PMWS (5, 7, 17, 19, 27, 45, 50, 61, 67, 81, 91, 104).

Recent genetic and phylogenetic analyses of PCV2 isolates revealed the existence of 2 different genotypes of PCV2: PCV2-group 1 and PCV2-group 2 (66). PCV2-group 1 and PCV2-group 2 isolates differ in length by 1 nt (1767 and 1768 nt, respectively), but exhibit no clear difference in pathogenesis between the two groups (66). PCV2-group 1 viruses separate into 3 clades, and group 2 viruses separate into 5 clades (66). Until recently, only PCV2-group 2 isolates were reported in the United States (15). In late 2005, outbreaks of PCV2 that caused much higher mortality of 5-50% in pigs were reported from pig farms in Kansas, North Carolina, and Iowa, and these outbreaks were found to be caused by PCV2-group 1 isolates (15).

1.2 Taxonomy

PCV1 and PCV2 belong to the virus family *Circoviridae* and genus *Circovirus* (99). The genus *Circovirus* includes PCV1, PCV2, beak and feather disease virus, canary cirvovirus, goose circovirus, and pigeon circovirus (99). Duck circovirus (38), finch circovirus (87), and gull circovirus (90) are tentative species in the genus. Circovirus has been detected in a number of other avian species including starlings (39), Australian ravens (92), ostrich (26), and pheasants (93). Another genus, *Gyrovirus*, is also classified in the *Circoviridae* family and contains chicken anemia virus (99), which has negative sense genomic organization and larger virions (19.1-26.5 nm) (32) .

Viruses in the genus *Circovirus* are most closely related to the plant virus family *Nanoviridae* due to shared stem loop structure at the origin of replication and the likeness of

encoded Rep proteins (55). These similarities also exist between circoviruses and the plant *Geminiviruses*, and it has been suggested that circoviruses are the genetic link between the geminiviruses and nanoviruses (63).

1.3 Genomic organization

The virions of PCV1 and PCV2 are nonenveloped, icosahedral, and range from approximately 17-21 nm in diameter (95). PCV1 and PCV2 genomes contain circular, single-stranded DNA of 1759 nt and 1768 nt, respectively (55, 95, 98), and have similar genomic organization with 2 main open reading frames (ORF), ORF1 and ORF2, which are encoded in the ambisense direction (Fig. 1.1).

ORF1 is highly conserved with approximately 83% nucleotide and 86% amino acid identity between PCV1 and PCV2 (60). ORF1 encodes the virus replication protein (Rep) (54) that is alternatively spliced into 5 associated RNAs in PCV2 and 8 associated RNAs in PCV1, of which only 2 are essential for virus replication, Rep and Rep' (13, 14, 53). ORF2 encodes the virus capsid (Cap) protein (62) and is more variable with approximately 67% nucleotide and 65% amino acid sequence identity between PCV1 and PCV2 (60). The main antigenic epitopes of the virus are located in the ORF2 Cap protein (30, 31, 44, 100). Recently a 3rd ORF, ORF3, was described in PCV2 (47), which is 315 bp in length and in the antisense direction within ORF1. The corresponding region of PCV1 encodes an ORF that is 612 bp in length, and only has 61.5% amino acid identity with ORF3 of PCV2 (47). It has been reported that the ORF3 protein of PCV2 causes apoptosis *in vitro* (47) and is involved in virus pathogenesis in mice (46).

1.4 Virus life cycle

1.4.1 Recognition, Attachment, and Entry into Target Cell

The mechanisms of PCV2 recognition, attachment, and entry into the target cells are not well understood. PCV2 antigen has been found in a number of different cells (22), indicating that PCV2 uses a commonly expressed cell receptor. It was recently determined that PCV2 binds to glycosaminoglycans (GAG), specifically heparan sulfate and chondroitin sulfate-B, on target cells as the first step of attachment (58). It is speculated that a co-receptor is required for viral entry since CHO cells, which lack GAG, can be infected with PCV2 (58). Entry of PCV2

virions and virus-like particles into the monocytic cells 3D4/31 was recently found to occur via clathrin-mediated endocytosis (59); it was further determined that endosomal acidification is an important requirement in PCV2 infection (59).

1.4.2 Genome Replication and Viral Gene Expression

PCV2 encodes so few proteins that it is believed the virus relies heavily on host machinery for virus gene expression and replication. PCV DNA synthesis requires DNA polymerase expressed during the S-phase of the cell cycle (97). Like geminiviruses, PCV replication is believed to occur via the rolling-circle method (52, 94), although experimental proof is lacking.

In vitro experiments in PCV2 infected PK-15 cells have shown that the first detected protein during virus replication was Cap, which was localized in the perinuclear region of infected cells (57). Subsequently, both the Cap and Rep proteins were detected at 12 hours post-inoculation (hpi) in the nucleus (57). Interestingly, the Cap protein was never found in the nucleus without the Rep protein, indicating that association with the Rep protein is required for Cap to enter the nucleus (57). By 36 hpi, the virus titer increased, signifying that the replication cycle was complete (57). Between 48 and 72 hpi, cells began detaching from the monolayers and Cap was abundantly detected in their cytoplasm (57). Similar protein expression patterns were found in porcine fetal cardiomyocytes and alveolar macrophages, but nuclearized antigens were detected later, at 48 hpi (57).

1.4.3 Capsid Formation, Virion Assembly, and Virus Release

There are no reports as to how the capsid forms, how the virion assembles, or how the virus is released from infected cells.

1.5 Pathogenesis

1.5.1 Entry of Virus and Incubation Period

PCV2 is transmitted by the oronasal route (82), which is substantiated by productive infection after intranasal inoculation of pigs (4, 9, 28, 40, 41, 79). It has been shown that PCV2 can also be transmitted transplacentally *in vivo*, although this does not appear to be a common

mode of transmission (51, 71, 72). In experimentally inoculated pigs, the incubation period ranges from 2 to 4 weeks (4, 9, 10, 36).

1.5.2 Viral Spread through Host

Initial replication of PCV2 occurs in lymphoid organs near the site of infection, such as the tonsil and lymph nodes (77), and also in B-cells (107). This leads to the speculation that PCV2 spreads via the lymphatic system, since virus has been detected in Peyer's patches, tonsil, lymph nodes, and spleen (16). Subsequent virus replication occurs in T lymphocytes and possibly in peripheral blood mononuclear cells (PBMC) (106, 107).

PCV2 viremia begins between 7 and 14 days post-inoculation in *in vivo* experiments, and virus is shed in nasal cavities, tonsillar and bronchial secretions, feces, and urine both in experimental *in vivo* infections and in field cases (10, 11, 41, 86, 88). Viral nucleic acid has been detected in semen by PCR in experimentally infected and healthy boars (33, 42).

Persistent PCV2 infection has been shown to occur. In field conditions, pigs up to 22 weeks of age had PCV2 DNA detected in their serum, although it is unknown if these pigs were persistently infected (76). *In vivo*, PCV2 DNA has been detected up to 71 days post-inoculation (DPI) (4, 9, 36, 41, 49, 73, 75, 79), and at 125 DPI in one pig (10).

1.5.3 Immune Response

It is uncertain if colostral antibodies provide protection against PCV2 infection. Some studies indicate that colostral antibodies are protective, since PMWS is not observed in pigs less than 4 weeks of age (2, 70). In contrast, Hassing et al (37) found that high levels of maternally derived antibody were not protective against PMWS.

IgG antibodies against PCV2 exist in pigs between 2-3 months of age in the field, yet many of those pigs become viremic suggesting that antibodies against PCV2 may not be fully protective (43, 76, 88). Pigs experimentally infected with PCV2 seroconvert between 14 and 28 DPI (4, 9, 40, 73). In specific pathogen free pigs, PCV2 serum neutralizing antibody was detected at 10 DPI, and the levels increased through 21 DPI, when 98% PCV2 neutralization was observed (56). In the field, PCV2 neutralizing antibody was detected at 10 weeks of age in Belgian pigs and 3 weeks of age in Danish pigs (56). There are differences in immune response to subclinical PCV2 infection and high levels of PCV2 replication which result in PMWS (56).

Pigs with PMWS had either low or undetectable levels of neutralizing antibody, as well as low IgM titers or antibody titers that quickly decreased when compared to their healthy group mates (56).

Recent research has shown that PCV2 modulates the immune system to its benefit. PCV2 DNA is able to inhibit the production of IFN-alpha by natural interferon producing cells (101). PCV2 also alters the immune response of PBMC during infection (20), and significantly diminishes the hosts' response to infection. It has been shown that dendritic cells harbor PCV2 antigen in their cytoplasm in the absence of cell modulation, indicating that these cells could be a source of virus for infection of other cells (102).

1.6 Diseases

Since its original link to PMWS, PCV2 has been associated with a number of other diseases including reproductive disease (105) and porcine dermatitis and nephropathy syndrome (PDNS) (89). An all inclusive term was recently suggested for these diseases: porcine circovirus-associated diseases (PCVAD) (1).

1.6.1 Reproductive Disease

Under experimental conditions, PCV2 infected the fetus via intrauterine inoculation and caused subsequent reproductive failure (105). It has been shown that the virus is transmitted transplacentally from mother to fetus, at which point it targets the heart (105). PCV2 has been detected in the heart, liver, lung, and kidney of stillborn fetuses by PCR, immunohistochemistry, and virus isolation (65, 105).

1.6.2 Porcine Dermatitis and Nephropathy Syndrome

In 1993, porcine dermatitis and nephropathy syndrome (PDNS), which affects the skin and kidney of pigs, was described in the United Kingdom (89). PDNS is characterized as a Type III hypersensitivity reaction, with immune complexes building up in the vascular and glomerular capillary walls (85). The clinical signs include red to purple lesions on the skin near the hindquarters, anorexia, depression, and mild fever (85). PCV2 antigen and/or nucleic acid have been detected in lymphoid tissues, kidney, liver, and skin of affected pigs (18, 23, 78). Porcine

reproductive and respiratory syndrome virus (PRRSV) has been identified as a co-infectious agent in some cases of PDNS (78).

1.6.3 Postweaning Multisystemic Wasting Syndrome

PCV2 is the primary causative agent of PMWS, which is characterized by severe progressive weight loss, dyspnea, lymph node enlargement, diarrhea, pallor, and jaundice in pigs 7-15 weeks of age (35, 60). Morbidity caused by PMWS ranges from 4-30% on affected farms, and 70-80% of those pigs die (84). In general, mortality associated with PMWS varies from 4 to 20% (84), but sporadic cases of PMWS also occur.

PMWS-affected pigs commonly have gross lesions including non-collapsed, tan-mottled lungs and enlargement of superficial inguinal, submandibular, mesenteric, and mediastinal lymph nodes (77). The majority of microscopic lesions occur in lymphoid tissues characterized by lymphocyte depletion, histiocytic inflammatory infiltration, and intracytoplasmic inclusion bodies (35, 77). In both field cases and *in vivo* experiments, B and T lymphocyte depletions have been observed in association with PMWS (21, 64, 77, 83), leading to the conclusion that the disease causes immune suppression. Microscopic lesions in the lungs are also common and include interstitial pneumonia and catarrhal purulent bronchopneumonia (77). PCV2 nucleic acid has been detected in a number of tissues including mesenteric, superficial inguinal, mediastinal, and submandibular lymph nodes, tonsils, Peyer's patches, lungs, spleen, intestinal mucosa, liver, and kidney (3, 27, 35, 77).

PMWS is difficult to reproduce *in vivo* and not all pigs infected with PCV2 develop PMWS in the field. It has been shown that co-infection with other pathogens is an important factor in the development of PMWS. Many pigs with PCV2 infection and severe PMWS often have additional infections including *Pneumocystis carinii* (80), porcine parvovirus and porcine pseudorabies virus (12). Co-infections with PCV2 and porcine parvovirus (68), or with PCV2 and *Mycoplasma hyopneumoniae* (69) have resulted in severe PMWS under laboratory conditions. It is speculated that these additional infections provide immune stimulation, up-regulate PCV2 replication, and enhance PMWS, although the mechanism has yet to be elucidated.

1.7 Prevention

1.7.1 Management Practices

Like porcine parvovirus, PCV2 is extremely difficult to inactivate. PCV2 is resistant to pasteurization at 60-80ºC for 30 min, dry-heat inactivation up to 120ºC for 30 min, pH 3, and chloroform treatment (8, 103). Therefore, good pig production practices are essential in preventing PCVAD and virus transmission. High-quality nutrition, ideal pen density, all-in-all-out flow, and removal of sick animals are important management practices (34). Pen cleanliness, suitable ventilation and temperature, and reduction of stress on pigs are also significant factors in disease management.

1.7.2 Vaccination

Due to the severity and variation of the diseases caused by PCV2 and ensuing difficulty for the pork industry, it is imperative to have an effective vaccine.

The first USDA-fully licensed PCV2 vaccine in the United States and Canada, Suvaxyn® PCV2 One Dose™, was a killed vaccine based upon a chimeric PCV1-2 virus developed by Fenaux et al (29). This chimeric PCV1-2 virus contains the immunogenic capsid gene of PCV2 in the genomic backbone of the non-pathogenic PCV1 (29). The Suvaxyn® PCV2 One Dose™ is intended for vaccination of piglets of 4 weeks and older for the prevention of PCV2 viremia and for the control of characteristic PMWS-associated pathological lesions. Early field studies of the Suvaxyn® PCV2 One Dose™, since its market release in July 2006, indicated that the vaccine is very effective: the mortality decreased from 8-10% in non-vaccinated pigs to 1.0-2.0% in vaccinated pigs. At least three other vaccines (recombinant baculovirus-based vaccines or killed whole PCV2 vaccine) will soon be available in the United States.

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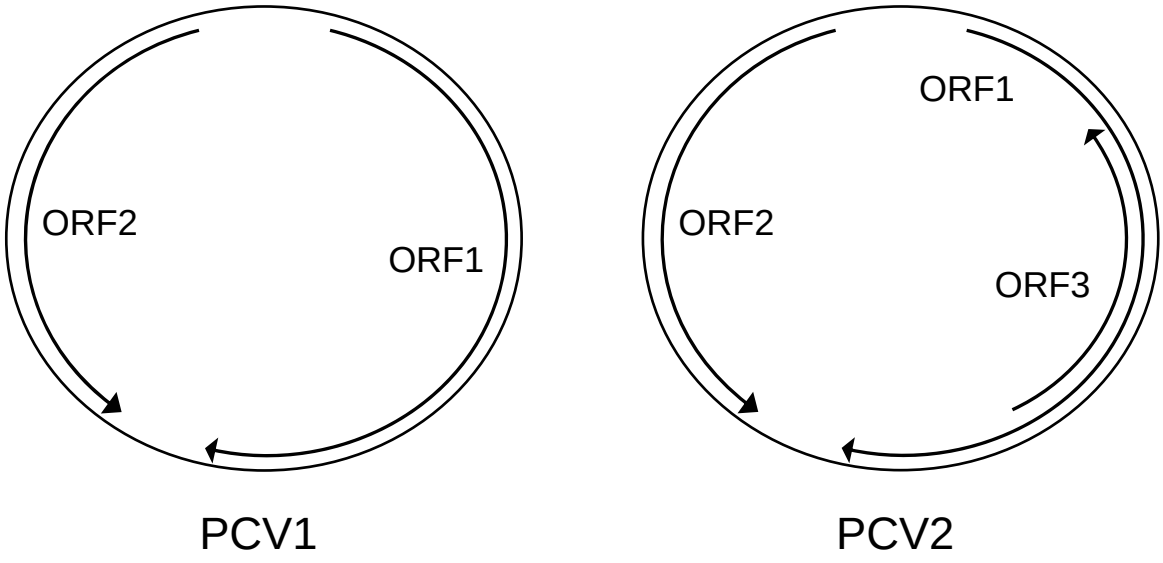

Fig. 1.1. Genomic organization of porcine circovirus type 1 (PCV1) and 2 (PCV2). PCV1 and PCV2 genomes contain circular, single-stranded DNA of 1759 nt and 1768 nt, respectively, and have similar genomic organization with 2 main open reading frames (ORF), ORF1 and ORF2, which are encoded in the ambisense direction. In both viruses, ORF1 encodes the virus replication genes and ORF2 encodes the virus capsid gene. PCV2 has a 3rd ORF, ORF3, which is reported to encode a protein responsible for apoptosis in PK-15 cells.

Chapter 2

Effects of type 2 porcine circovirus (PCV2) maternal antibodies on experimental infection of piglets with PCV2

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2.1 Abstract

Porcine circovirus type 2 (PCV2), the primary causative agent of postweaning multisystemic wasting syndrome (PMWS), is widespread in swine herds and most pigs are seropositive. Neonates have variable levels of maternally derived PCV2 antibodies. The objective of this study is to determine the effects of PCV2 maternal antibodies on and response to experimental PCV2 infection. Twenty-four piglets of 2 weeks of age were divided into 4 groups based on ELISA levels of PCV2 maternal antibodies and each group was housed in a separate room: group A (n=6, sample/positive [S/P] ratio <0.2, negative), group B (n=5, S/P ratio >0.2-<0.5, low), groups C (n=8) and D (n=5) (S/P ratio >0.5, high). Piglets in groups A, B, and C were all inoculated with PCV2 at day 0, and subsequently challenged with a homologous PCV2 virus 42 days later. Group D piglets were not exposed to PCV2 at day 0 but were challenged along with all pigs in other groups 42 days later. All pigs were necropsied at 63 days post-inoculation (DPI: corresponds to 21 days post-challenge, DPC). ELISA was used to assess PCV2 antibody level and quantitative PCR was used to determine serum viral DNA load. Following the initial inoculation and before challenge at 42 DPI, seroconversion to PCV2 antibodies occurred in 5/6 group A piglets, and antibody rose above 0.2 S/P ratio cutoff following decay below the threshold in 1/5 group B piglets. Viremia was detected in 5/6 piglets from group A, 4/5 pigs from group B, and 2/8 pigs in group C. After challenge at 42 DPI, PCV2 DNA was detectable from 7-21 DPC in the sera of 6/6 group A pigs, 4/5 group B pigs, 3/8 group C pigs, and 5/5 group D pigs. The results from this study indicated that maternal antibodies are

not fully protective against PCV2 infection. The data have important implications for selecting the optimal timing of vaccination with a live PCV2 vaccine and for the management of infected herds.

2.2 Introduction

Porcine circovirus was initially isolated as a persistent contaminant of the porcine kidney PK-15 cell line (ATCC CCL-33) (47). It has been shown that PCV is an ubiquitous virus that does not cause any disease in piglets (5, 8, 11, 12, 46). Recently, a new swine disease, designated postweaning multisystemic wasting syndrome (PMWS), was recognized in piglets in Canada (13, 20). Subsequently, a variant strain of PCV has been shown to be the primary causative agent of PMWS (1, 4, 13). The PMWS-associated PCV was designated PCV2, whereas the non-pathogenic PK-15 cell derived PCV was designated PCV1 (32).

PCV2 belongs to the family *Circoviridae*, which includes psittacine beak and feather disease virus (BFDV), chicken anemia virus (CAV), and, tentatively, pigeon circovirus (PiCV) (48). PCV2 is an icosahedral, nonenveloped, circularized, single strand DNA virus of 1,768 bp (19, 30, 32). The ORF2 gene of PCV2 encodes the major capsid protein that contains neutralizing epitopes (28, 35, 49), whereas the ORF1 gene encodes Rep proteins that are involved in virus replication (31). PCV2 has been isolated from both diseased and healthy piglets in almost all swine producing countries (1, 4, 6, 7, 19, 22, 25, 27, 30, 32, 33, 36, 38, 43, 45). The genetic determinant for PCV2 virulence is not known (10), although two amino acids in the capsid gene have been shown to be involved in PCV2 attenuation (17).

PMWS mainly affects 5-16 week old pigs (6, 9). Morbidity associated with PCV2 infection and PMWS ranges from 5-30%, while case mortality can be up to 50% (1, 6, 20). The characteristic symptoms of PMWS include progressive weight loss, dyspnea, enlargement of lymph nodes, diarrhea, pallor, and jaundice (20). The hallmark pathological lesion of PCV2 infection and PMWS is lymphoid depletion and histiocytic replacement of lymphoid follicles (37). Piglets co-infected with PCV2 and porcine parvovirus, PCV2 and porcine respiratory and reproductive syndrome virus (PRRSV), or PCV2 and *M. hyopneumoniae* have more severe clinical disease and PCV2-associated lesions (3, 6, 21, 23, 24, 26, 39, 40, 42, 44). A vaccine against PMWS and PCV2 is not yet available, although an experimental vaccine based on a chimeric virus between PCV1 and PCV2 is very promising (14-16, 18).

Passively acquired antibodies generally confer protection against homologous viral infections to newborn piglets. Under field conditions, PCV2 infection is widespread and most breeding age pigs are seropositive (41). The window of time over which passive antibodies decay is wide and variable (41). The objective of this study was to assess the role of PCV2

maternal antibodies in preventing PCV2 infection in piglets. We hypothesize that the higher the level of PCV2 maternal antibody, the more protection the piglets will have against PCV2 infection.

2.3 Materials and Methods

2.3.1 PCV2 virus

To generate an infectious PCV2 virus stock, PK-15 cells were transfected with plasmid containing the PCV2 infectious DNA clone, as previously described (Appendix A, 15, 18). Three days after transfection, the virus was harvested by freezing and thawing the cells three times. The PCV2 used for the construction of the PCV2 infectious DNA clone was originally isolated from a piglet with clinical PMWS (14). The PCV2 virus generated from the infectious DNA clone produced pathological lesions characteristic of PMWS (15).

2.3.2 Infectivity titration of the PCV2 virus stock

To determine the infectivity titer of the PCV2 virus stock, PK-15 cells were grown on 8 well Lab-Tek chamber slides. When the cells reached approximately 70% confluency, 200 µl of each of the 10-fold diluted PCV2 virus stock (10^{-1} to 10^{-6}) was each inoculated onto 4 duplicated wells. After 1 hour incubation at 37°C, the infected cells were maintained with minimal essential media with 2% fetal bovine serum and 1X antibiotic/antimycotic. Indirect immunofluoresence assay (IFA) with PCV2-specific polyclonal rabbit antibody was performed to determine the infectivity titer by the Karber-Spearman method (Appendix A, 16).

2.3.3 Selection of piglets with various levels of PCV2 maternal antibodies

A total of 106 specific-pathogen-free (SPF) piglets that were 12 days of age were acquired from a commercial source. All piglets were tested with a PCV2-specific ELISA for the presence of maternal antibody prior to grouping (34). Of the 106 piglets tested, 24 piglets were selected for this study. Piglets were separated into 4 different groups based on the level of maternal antibodies detected 2 days prior to experimental exposure to PCV2. Six piglets with maternal antibodies at a sample/positive (S/P) ratio <0.2 were placed in group A; five piglets with antibodies at a S/P ratio between 0.2 and 0.5 were placed in group B. Of the 13 pigs with antibodies at a S/P ratio >0.5, eight were assigned to group C, and five were assigned to group D

(Table 2.1). The ELISA S/P ratio cutoff is determined to be 0.2 (34), therefore piglets at a S/P ratio <0.2 are considered negative for maternal antibodies. Piglets with a S/P ratio of >0.2 to <0.5 are designated as having a low level of detectable maternal antibodies, whereas piglets with a S/P ratio of >0.5 are considered as having a high level of detectable maternal antibodies (34).

2.3.4 Experimental infection

Piglets from groups A, B, and C (Table 2.1) were all exposed to PCV2 at day 0: each received 3 ml (1 x $10^{3.55}$ $TCID_{50}$) of the PCV2 live virus inoculum by slow instillation into the nasal cavity. Piglets in group D were not exposed to PCV2 at day 0 (Table 2.1). All piglets were bled prior to inoculation and weekly thereafter. The sera were tested for anti-PCV2 antibodies by ELISA (34), and for PCV2 DNA by modified quantitative PCR (16, 34).

Piglets in groups A, B, C, and D were all challenged with 3 ml (1 x $10^{4.7}$ $TCID_{50}$) of a homologous PCV2 at 42 days post-inoculation (DPI), at which time the piglets were 9-10 weeks of age. Approximately 1 ml of inoculum was given intramuscularly and 2 ml intranasally. All piglets were bled weekly after challenge and necropsied at 21 days post-challenge (DPC: corresponds to 63 DPI).

2.3.5 ELISA

Antibody levels to PCV2 in sera were determined by ELISA as previously described (34) at Iowa State University Diagnostic Laboratory. A S/P ratio < 0.2 is considered negative (29).

2.3.6 Quantitative PCR

Viremia and PCV2 virus DNA load in sera were determined by a modified quantitative PCR (16, 29). Briefly, viral DNA was extracted from 200 µl of each serum sample with Qiagen's QIAamp DNA Mini Kit according to the manufacturer's protocol. DNA was eluted in 100 µl of elution solution for all samples. The primers (MCV1 and MCV2) used for the quantitative PCR were based on known sequences of PCV2 and PCV1 (14, 16, 18). Each PCR reaction consisted of 0.75 µl MCV1 and MCV2 primers, 10 µl of template, 12.5 µl of iQ SYBR Green supermix, and 1 µl of DNase-RNase-proteinase-free water for a total volume of 25 µl. All reactions were run in triplicate. A standard dilution series with a known amount of plasmid containing a single copy of the PCV2 genome was run simultaneously with samples in each

reaction (16). The PCR parameters consisted of a 95ºC initial enzyme activation step for 3 min, followed by 38 cycles of a 10 s denaturation at 95ºC, 15 s annealing at 53ºC, and 10 s extension at 72ºC. After amplification, a melt curve analysis was performed to assure the correct product was formed. Quantification of viral genomic copies per ml (GC/ml) of serum was then calculated (16).

2.3.7 Statistical Analysis

S/P ratios of PCV2 antibody and PCV2 viral DNA loads were compared and evaluated by simple T-test, and analysis of variance and regression analysis using the TTEST and GLM procedures of the SAS (version 9.1, SAS Institute Inc., Cary, NC).

2.4 Results

2.4.1 Effect of maternal antibodies on experimental PCV2 infection

Piglets in group A were all considered negative (S/P ratio <0.2) for PCV2 maternal antibodies 2 days prior to experimental inoculation. After exposure to PCV2, seroconversion started at 35 DPI in 4/6 of these piglets. By 42 DPI, 5/6 piglets in this group had seroconverted (Fig. 2.1). Two of the six piglets in group A had an onset of viremia at 14 DPI with a viral DNA load ranging from 10^5-10^6 GC/ml serum (Table 2.2). All but one piglet had viremia by 42 DPI at the time points tested. Clinical symptoms of PMWS were not evident.

The piglets in group B all had a low level of maternal antibody (S/P >0.2 to <0.5) 2 days prior to inoculation. The low levels of maternal antibodies waned by 14 DPI. On 21 DPI, the 5 piglets in this group had become seronegative (S/P ratio <0.2) and remained so through 35 DPI. At 42 DPI, one pig (pig #71) seroconverted and the other 4 pigs were still seronegative (Fig. 2.1). Viremia was first detected in two of the five piglets at 21 DPI with a viral DNA load of 10^6 GC/ml serum. Three piglets had viremia by 42 DPI with a viral DNA load ranging from 10^5 to 10^6 GC/ml serum.

Piglets in group C all had high levels of maternal antibodies 2 days prior to experimental PCV2 exposure. The maternal antibodies gradually waned from 7 to 42 DPI, and there was no rise of antibody titer between 7 and 42 DPI in any of the piglets in this group (Fig. 2.1). Viremia was detected in 1/8 piglet (#15) at 21 DPI with a viral DNA load of 10^4 GC/ml serum (Table

2.2). By 42 DPI, only two piglets in group C had viremia with a viral DNA load ranging from 10^4 to 10^5 GC/ml serum.

The mean -2 DPI antibody level in piglets that became infected with PCV2 was lower (S/P ratio=0.37, standard deviation=0.328) than in piglets that did not become infected (S/P ratio=0.84, standard deviation=0.515) (P = 0.044). The day of peak viremia in infected piglets was not related to initial maternal antibody level (P = 0.50), but peak viremia level decreased with increasing maternal antibody level at -2 DPI (P = 0.025).

2.4.2 Effect of maternal antibody decay and prior PCV2 exposure on PCV2 challenge

To determine the length of protection that PCV2 maternal antibodies can confer to the piglets, and to assess the outcome of prior PCV2 exposure on re-infection of piglets by PCV2, we challenged all piglets with a homologous PCV2 at 42 DPI, at which time the piglets were approximately 9-10 weeks of age. At the time of challenge, 5/6 pigs in group A were seropositive in response to the initial PCV2 exposure at DPI 0 (Fig. 2.1), and 4/6 piglets were viremic at challenge (Table 2.2). The maternal antibodies in group B piglets all fell below the S/P ratio cut-off value by 21 DPI, and all but one piglet were seronegative (Fig. 2.1) and 4/5 piglets were viremic at the time of challenge at 42 DPI (Table 2.2). In group C, at the time of challenge at 42 DPI, 4 piglets were still positive for PCV2 maternal antibodies with S/P ratio ranging from 0.23 to 0.98, the S/P ratios in the other 4 pigs were all <0.2 (Fig. 2.1), and 2 piglets were viremic at challenge (Table 2.2). After challenge, PCV2 antibody levels in piglets from groups A and B continue to rise. In contrast, there was no detectable rise of PCV2 antibody level in group C piglets after challenge.

After challenge, all six piglets in group A were viremic and had serum viral DNA loads ranging from 10^5 to 10^9 GC/ml serum at 7 DPC, and from 10^4 to 10^7 GC/ml serum at 21 DPC. In group B piglets, 4/5 piglets remained viremic and had serum viral DNA loads ranging from 10^4 to 10^7 GC/ml serum at 7 DPC (Table 2.2). By 21 DPC, the serum viral DNA load in group B piglets ranged from 10^4 to 10^6 GC/ml serum. After challenge, two group C piglets remained viremic with no change in the range of serum viral DNA load and only one additional piglet, which had a very low S/P ratio at the time of challenge, developed viremia (Table 2.2).

In group D, the piglets were not exposed to PCV2 until about 2 months of age (Table 2.1). The PCV2 maternal antibody waned gradually over the course of the experiment (Fig. 2.1). At the time of challenge, all 5 pigs in group D had low levels of maternal antibodies with S/P ratios ranging from 0.24 to 0.33. After challenge, there was no detectable rise of PCV2 antibody titer (Fig. 2.1). However, all five piglets in group D developed viremia with a serum viral DNA load ranging from 10^5 to 10^6 GC/ml serum at 14 DPC.

2.5 Discussion

PCV2 is widespread in the pig population and most breeding age animals are seropositive. Consequently neonate piglets have variable levels of PCV2 maternal antibodies. Postweaning multisystemic wasting syndrome, caused by PCV2, mainly affects young piglets of 5-16 weeks of age (2, 9). Therefore, it is important to understand the role of PCV2 maternal antibodies in protecting neonate piglets from PCV2 infection.

Our study showed that presence of low level of PCV2 maternal antibodies does not protect young piglets from experimental PCV2 infection. When exposed to PCV2, piglets with no or low levels of maternal antibodies became infected and viremia is detectable in 9/11 group A and B piglets. High levels of PCV2 maternal antibodies confer only partial protection against PCV2 infection, as viremia is detected in 2/8 group C piglets (Fig. 2.1, Table 2.2), but peak viremia levels in piglets were reduced with higher antibody levels at inoculation (P=0.025). When piglets in group D were challenged at a later time at 42 DPI, 5/5 piglets became infected. Taken together, the data from this study indicate that low levels of PCV2 maternal antibodies are not protective against PCV2 infection, whereas high levels of PCV2 maternal antibodies are only partially protective. The results from this study could explain why many neonate piglets born to PCV2 positive sows are still susceptible to PCV2 infection in swine farms.

We also attempted to assess the effect of prior PCV2 infection on challenge by the same virus. The PCV2-exposed piglets with no or low level of maternal antibodies (groups A and B) are not protected from the homologous challenge by PCV2, as evidenced by continuous viremia despite rising antibody levels. At the time of challenge (about 2 months of age), 5/6 piglets in group A already developed active PCV2 antibody response from the initial exposure. It is likely that both humoral immune response and cell-mediated immunity (CMI) are required for full

protection (16). Future studies with PCV2 challenge beyond 42 DPI are needed to fully evaluate the effects of prior PCV2 exposure on homologous PCV2 challenge.

In conclusion, the results from this study showed that the levels of PCV2 maternal antibodies are an important determinant of a piglet's response to PCV2 infection. It appears that the higher the level of maternal antibody, the more protection the piglet will have. Unfortunately, even in piglets with high levels of PCV2 maternal antibodies, the protection is not complete. The data from this study have important implications for selecting the optimal timing of vaccination, especially with a live PCV2 vaccine when it becomes available.

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Fig. 2.1.

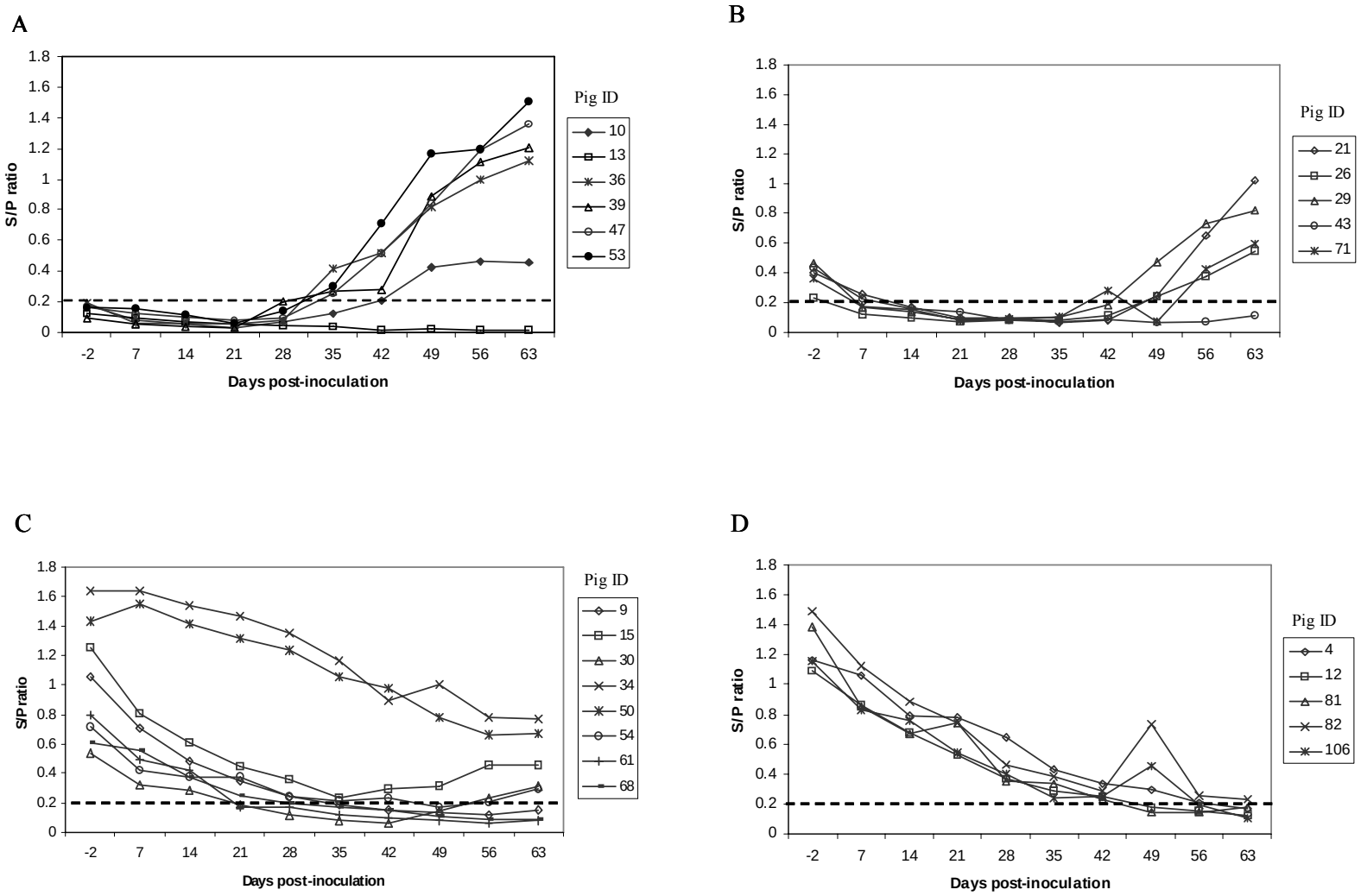
A
S/P ratio
Days post-inoculation
Pig ID
10
13
36
39
47
53
B
S/P ratio
Days post-inoculation
Pig ID
21
26
29
43
71
C
S/P ratio
Days post-inoculation
Pig ID
9
15
30
34
50
54
61
68
D
S/P ratio
Days post-inoculation
Pig ID
4
12
81
82
106

Fig. 2.1. Kinetics of PCV2 antibody in response to infection as well as PCV2 maternal antibody decay for each group of piglets throughout the experiment. Piglets in groups A, B, and C were exposed to PCV2 at day 0 (DPI). Piglets in group D were not exposed to PCV2 at day 0 but were challenged with a homologous PCV2 42 days later, along with all piglets in the other groups. Assignment of piglets to groups based on maternal antibody levels prior to inoculation with PCV2 was as follows: group A: n=6, negative maternal antibody (S/P <0.2); group B: n=5, low level of maternal antibodies (S/P >0.2-<0.5); groups C (n=8) and D (n=5): high level of maternal antibodies (S/P >0.5).

Table 2.1. Experimental design to assess the effect of PCV2 maternal antibodies on experimental infection with PCV2

Group	No. of pigs	Maternal Antibody Level	PCV2 inoculation 3 weeks of age 0 DPI [a]	PCV2 challenge 9 weeks of age 42 DPI/0 DPC [b]	Necropsy 12 weeks of age 63 DPI (21DPC)
A	6	Negative (<0.2)	PCV2	PCV2	6
B	5	Low (>0.2-<0.5)	PCV2	PCV2	5
C	8	High (>0.5)	PCV2	PCV2	8
D	5	High (>0.5)	None	PCV2	5

[a]DPI, days post-inoculation.
[b]DPC, days post-challenge.

Table 2.2. Serum viral DNA loads (genomic copy per ml serum) in pigs throughout the study as detected by quantitative PCR

		DPI[a]						Challenge	DPC[b] (DPI)		
	Pig ID	0	7	14	21	28	35	0 (42)	7 (49)	14 (56)	21 (63)
Group A: Negative maternal antibody (S/P <0.2)	**10**	-	-	-	$2.6x10^7$	$1.4x10^7$	$1.2x10^6$	$3.2x10^6$	$3.4x10^6$	$1.2x10^6$	$4.7x10^6$
	13	-	-	-	-	-	-	-	$2.5x10^5$	$1.3x10^6$	$2.0x10^5$
	36	-	-	$3.0x10^6$	$5.5x10^8$	$3.7x10^8$	$3.1x10^7$	$2.56x10^8$	$1.8x10^9$	$1.1x10^7$	$1.5x10^7$
	39	-	-	-	$6.3x10^8$	$2.4x10^7$	$6.2x10^6$	-	$1.4x10^6$	$1.2x10^5$	$8.7x10^5$
	47	-	-	$1.3x10^5$	$5.0x10^5$	$1.9x10^6$	$5.3x10^4$	$1.7x10^5$	$2.0x10^5$	$3.0x10^5$	$2.9x10^5$
	53	-	-	-	$2.8x10^8$	$6.3x10^6$	$1.0x10^9$	$7.8x10^5$	$3.0x10^5$	$2.4x10^7$	$5.6x10^4$
No. pigs with viremia[c]		**0/6**	**0/6**	**2/6**	**5/6**	**5/6**	**5/6**	**4/6**	**6/6**	**6/6**	**6/6**
Group B: Low maternal antibody (S/P >0.2-<0.5)	**21**	-	-	-	-	$1.0x10^7$	$3.5x10^7$	$2.3x10^6$	$5.8x10^7$	$3.5x10^8$	$6.0x10^5$
	26	-	-	-	$4.7x10^6$	$7.5x10^4$	$8.7x10^4$	$1.7x10^6$	$5.4x10^5$	$1.9x10^5$	$8.6x10^4$
	29	-	-	-	$4.6x10^6$	$5.8x10^6$	$2.5x10^5$	$1.3x10^6$	$1.4x10^6$	$2.2x10^6$	$2.6x10^6$
	43	-	-	-	-	$3.3x10^4$	$5.5x10^4$	$8.4x10^5$	$9.6x10^4$	$3.2x10^4$	$1.5x10^6$
	71	-	-	-	-	-	-	-	-	-	-
No. pigs with viremia		**0/5**	**0/5**	**0/5**	**2/5**	**4/5**	**4/5**	**4/5**	**4/5**	**4/5**	**4/5**
Group C: High maternal antibody (S/P >0.5)	**9**	-	-	-	-	-	-	-	-	-	-
	15	-	-	-	$1.9x10^4$	$2.3x10^5$	$5.6x10^4$	$3.6x10^4$	$3.6x10^4$	$9.8x10^4$	-
	30	-	-	-	-	$2.7x10^5$	$1.7x10^4$	$3.3x10^5$	$3.3x10^5$	$3.3x10^5$	$7.5x10^4$
	34	-	-	-	-	-	-	-	-	-	-
	50	-	-	-	-	-	-	-	-	-	-
	54	-	-	-	-	-	-	-	-	-	-
	61	-	-	-	-	-	-	-	$9.2x10^5$	$4.3x10^5$	$1.5x10^5$
	68	-	-	-	-	-	-	-	-	-	-
No. pigs with viremia		**0/8**	**0/8**	**0/8**	**1/8**	**2/8**	**2/8**	**2/8**	**3/8**	**3/8**	**2/8**
Group D: High maternal antibody (S/P >0.5)	**4**	-	-	-	-	-	-	-	-	-	$1.1x10^6$
	12	-	-	-		-	-	-	-	$3.9x10^5$	-
	81	-	-	-	-	-	-	-	-	$2.5x10^5$	$2.1x10^4$
	82	-	-	-	-	-	-	-	-	$7.5x10^6$	$3.9x10^5$
	106	-	-	-	-	-	-	-	-	$1.2x10^5$	-
No. pigs with viremia		**0/5**	**0/5**	**0/5**	**0/5**	**0/5**	**0/5**	**0/5**	**0/5**	**4/5**	**4/5**

[a] DPI, days post-inoculation.
[b] DPC, days post-challenge.
[c] Number of pigs with viremia/total number of pigs in the group.

Chapter 3

Evaluation of the roles of P110A and R191S mutations on the capsid protein of porcine circovirus type 2 (PCV2) in virus attenuation

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3.1 Abstract

Porcine circovirus type 2 (PCV2) is the causative agent associated with postweaning multisystemic wasting syndrome (PMWS) in pigs. Previously, our lab reported two nucleotide mutations resulting in 2 amino acid substitutions in the PCV2 capsid protein that enhanced virus replication *in vitro* and attenuated the virus *in vivo*. To determine if these two substitutions, P110A and R191S, act singly or collectively in altering PCV2 virulence, we constructed 2 single mutants (P110A and R191S) and a double mutant (DM), and evaluated their pathogenicity in pigs. Group 1 pigs (n=8) were inoculated with PBS buffer and served as negative controls, and group 2 pigs (n=12) were inoculated with wild-type PCV2 (PCV2). Groups 3, 4, and 5 pigs (n=12, each) were inoculated with P110A, R191S, and DM, respectively. Infected and control pigs were assessed for weight changes, rectal temperature, respiratory scores, PCV2 antibodies, PCV2 viral load, and macroscopic and microscopic lesions. At 21 days post-infection (DPI), pigs inoculated with P110A had more significant lymphoid depletion (P=0.006) and histiocytic replacement (P=0.02) of follicles in the lymph nodes than those infected with DM, and more significant lymph node lymphoid depletion than pigs infected with R191S (P=0.02). At 42 DPI, PCV2-infected pigs had significantly more severe histiocytic replacement of follicles in the lymph nodes and lymphohistiocytic enteritis than pigs infected with any of the mutants (P<0.02). PCV2-infected pigs also had significantly more severe lymph node lymphoid depletion (P=0.007) and PCV2 antigen in lymph nodes (P=0.02) than P110A-infected pigs at 42 DPI. The data suggest that both substitutions are necessary for PCV2 attenuation.

3.2 Introduction

Postweaning multisystemic wasting syndrome (PMWS) was first recognized in 1991, and has become an economically important global disease in the pig industry (2, 6, 7, 15). Porcine circovirus type 2 (PCV2) is the primary causative agent of PMWS (1, 2, 7). Clinically, PMWS is characterized by wasting, diarrhea, increased respiratory rate, and jaundice (15). Lymphoid depletion and histiocytic replacement of lymphoid follicles are the main microscopic features of PCV2 infection and PMWS (29). Enlargement of lymph nodes is a major macroscopic feature of PMWS (15). Pigs between the ages of 5-16 weeks are typically affected by PMWS (3, 6). High levels of maternal antibodies can prevent PCV2 infection in young pigs, but lower levels are generally not protective (25). Case mortality rate associated with PCV2 infection and PMWS can be up to 50%, while morbidity ranges from 5-30% (1, 3, 15).

PCV2 is a nonenveloped icosahedral virion, and the genomic DNA is ambisense, single stranded, and approximately 1.7 kb in length (14, 22, 26). PCV2 belongs to the family *Circoviridae* along with porcine circovirus type 1, chicken anemia virus, psittacine beak and feather disease virus, columbid circovirus, goose circovirus, canary circovirus, and duck circovirus (4, 16, 23, 31, 37, 38). The PCV2 genome has two main open reading frames (ORF); ORF 1 encodes one RNA transcript that is alternatively spliced into 4 additional transcripts that together code for the ORF 1 Rep proteins responsible for viral replication, and ORF 2 encodes the capsid protein which is transcribed from a single RNA transcript (5, 24, 28). Recently, another ORF, ORF 3, was reported in association with apoptosis induction (19). Apoptosis has been previously examined as a potential cause of lymphocyte depletion associated with PCV2 infection, but no consensus has been reached on this issue (17, 21, 33, 34).

The ORF2 capsid protein contains the main neutralizing epitopes (10, 11, 18, 39). A chimeric vaccine using PCV2 ORF 2 as the antigen in porcine circovirus type 1 genomic backbone was recently developed and shown to confer protection to piglets challenged with PCV2 (10). By epitope mapping of the capsid protein, Lekcharoensuk et al. (18) identified three antigenic epitopes in amino acids 47 to 84, 165 to 200, and the last 4 amino acids, while Truong et al. (39) identified a B cell epitope by ELISA in amino acids 117 – 131 of the capsid protein. Our lab recently reported that two amino acid substitutions in the capsid protein of PCV2 slightly enhanced virus replication *in vitro* and attenuated the virus *in vivo* (11). The substitutions R191S with nt 573 mutated from an A to a C and P110A with nt 328 mutated from a C to a G were both

found in the capsid gene after serially passaging the virus 120 times in PK-15 cells (11). The R191S substitution was found late, between passages 91 and 120, while P110A was found at passage 30 (11). Pigs infected with the passage 120 PCV2 containing both substitutions had significantly lower levels of PCV2 viremia and PMWS-associated microscopic lesions compared to pigs infected with wild-type PCV2 (11), indicating attenuation.

The objective of this study was to determine if the two amino acid substitutions in the capsid protein (R191S and P110A) act singly or collectively in PCV2 attenuation. We hypothesize that the P110A and R191S substitutions act collectively in the attenuation of PCV2. Three PCV2 mutants containing either one of the two substitutions or both substitutions were created and their pathogenicity was compared *in vivo*. We report here that both substitutions are necessary for PCV2 attenuation.

3.3 Materials and Methods

3.3.1 Construction of single mutants R191S and P110A, and double mutant DM

Site-directed mutagenesis was performed on the PCV2 infectious DNA clone using Stratagene's Quikchange Multi Site-Directed Mutagenesis Kit (La Jolla, CA) (9) to produce all three mutants: R191S mutant with nt 573 mutated from an A to a C; P110A mutant with nt 328 mutated from a C to a G; and a double mutant (DM) containing both mutations (Fig. 3.1). Primer 673.Mut.insert (5' CTACAAACCTCTAGCAATGTGGACCACGTAGGCC 3') was used to mutate nucleotide 573 to generate R191S single mutant and primer 918.Mut.insert (5' GGCCCTGCTCCGCCATCACCCAGG 3') was used to mutate nucleotide 328 to produce P110A single mutant. The double mutant DM was generated with both primers 673.Mut.insert and 918.Mut.insert. For each reaction, the mutant clone was synthesized with thermocycling using 95°C for an initial denaturation, followed by 30 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 58°C, and extension for 20 min at 65°C. The PCR products (plasmid mutants) were digested with *Dpn*I for selection of ssDNA and then transformed into TOP10 cells. The white colonies were cultured and analyzed by *Sac*II restriction digestion. The mutant clones were sequenced for both strands with the primers Upper 1 (5' GGTTACACGGATATTGTAGTCCTG 3') and Lower 1 (5' ATTTAATATTGACGACTTTGTTCC 3') to confirm the introduced mutations.

3.3.2 Preparation of virus inocula for *in vivo* study

The full-length viral genomic DNA of wild-type PCV2, single mutants P110A and R191S, and double mutant DM were each excised from the pBluescript II SK+ plasmid vector using the restriction enzyme *Sac*II (8). The digestion products were run on a 1% agarose gel and the 1.7 kb virus genomic DNA bands were excised, purified using the GeneClean Spin Kit (Q-biogene Inc., Morgan Irvine, CA), and quantified on a 1% agarose gel using molecular weight ladder, Hyperladder I (Bioline USA Inc., Randolph, MA). T4 DNA ligase (Stratagene Corp.) was used to concatomerize the full-length purified virus genomic DNA in an overnight ligation reaction at 4°C. Twenty-four T-25 flasks of 70% confluent PK-15 cells were transfected with approximately 12 μg of each concatomerized viral genomic DNA (6 flasks per mutant) using Lipofectamine and Plus Reagent (Invitrogen Inc., Carlsbad, CA), essentially as previously described (9). Three days post-transfection, the virus was harvested by freezing and thawing the transfected cells three times, and the infectious titer of the virus stock was determined by IFA with PCV2-specific antibody as previously described (9).

3.3.3 *In vivo* study to assess the effects of the mutants on virus pathogenicity

Fifty-six specific pathogen free pigs were randomly assigned to 5 groups. Eight pigs in group 1 served as negative controls. The other 48 pigs were randomly divided into 4 groups of 12 pigs each. Each group of pigs was housed in a separate room on raised wire decks. At 3 weeks of age, pigs in group 1 were each sham-inoculated with saline buffer, group 2 pigs were each inoculated with wild-type PCV2, group 3 with DM, group 4 with P110A, and group 5 with R191S. All pigs in groups 2-5 were inoculated with 1.0×10^5 $TCID_{50}$/ml of the respective virus (2 ml intranasally and 1 ml intramuscularly).

3.3.4 Clinical evaluation of pigs

The rectal temperature and respiratory scores of each pig were recorded at 0 DPI and every other day thereafter. Respiratory scores ranged from 0-6; 0= normal, 1= mild dyspnea and/or tachypnea when stressed, 2= mild dyspnea and/or tachypnea when at rest, 3= moderate dyspnea and/or tachypnea when stressed, 4= moderate dyspnea and/or tachypnea when at rest, 5= severe dyspnea and/or tachypnea when stressed, and 6= severe dyspnea and/or tachypnea

when at rest (13). The weight of each pig was recorded at 0 DPI and weekly thereafter until 42 DPI.

3.3.5 Necropsy and gross pathology

Half of the pigs in each group were necropsied at 21 days post-inoculation (DPI) and the remaining pigs were necropsied at 42 DPI. Macroscopic lung lesions were scored (0-100% of the lung affected) and tracheobronchial, mesenteric, external iliac, mediastinal, and superficial inguinal lymph nodes were assessed for change in size as previously described (13, 30). Sections of lung, lymph nodes (superficial inguinal, mediastinal, tracheobronchial, mesenteric), spleen, tonsil, liver, kidney, and small and large intestine were collected at necropsy, fixed in 10% neutral-buffered formalin, and processed for histological examination as previously described (30).

3.3.6 Histopathological examination of tissues

Microscopic lesions were evaluated in a blinded fashion. Lung lesions were scored for presence and severity of interstitial pneumonia ranging from 0= normal to 6= severe as previously described (13). Lymphoid tissues (lymph nodes, tonsil, spleen) were scored for the presence of lymphoid depletion ranging from 0= normal to 3= severe lymphoid depletion with loss of lymphoid follicle structure and for the presence of inflammation ranging from 0= normal to 3= severe histiocytic-to-granulomatous inflammation with replacement of follicles (30). Sections of heart, liver, kidney, ileum and colon were evaluated for the presence of inflammation ranging from 0= normal to 3= severe (13).

3.3.7 Immunohistochemistry of lymph nodes, tonsil, and spleen

Immunohistochemistry for detection of PCV2-specific antigen was done on formalin-fixed and paraffin-embedded sections of spleen, lymph nodes, and tonsil as previously described (35). Assessment of the amount of PCV2-antigen was done in a blinded fashion and the score ranged from 0= no staining detectable to 3= large numbers of PCV2-antigen diffusely distributed in the tissue section (30).

3.3.8 Serology

Blood samples were collected at 1 day prior to inoculation and weekly thereafter until 42 DPI. IgG antibodies to PCV2 in sera were determined by ELISA as previously described (27) at Iowa State Univeristy Diagnostic Laboratory. A sample/positive (S/P) ratio > 0.2 is considered positive (27).

3.3.9 Quantitative polymerase chain reaction (Q-PCR)

Lymph nodes from 21 and 42 DPI and all weekly sera were tested for PCV2 virus DNA load with a modified quantitative PCR (10, 20). Tracheobronchial lymph nodes were homogenized at 1% (weight/volume) in cold PBS buffer. Viral DNA was extracted from 200 µl of each lymph node homogenate and serum sample with Qiagen's QIAamp DNA Mini Kit according to the manufacturer's protocol, and eluted in 100 µl of elution solution. The primers (MCV1 and MCV2) used for the quantitative PCR were based on known sequences of PCV2 and PCV1 (8, 10, 12). Each PCR reaction consisted of 0.75 µl MCV1 and MCV2 primers, 10 µl of template, 12.5 µl of iQ SYBR Green Supermix, and 1 µl of DNase-RNase-proteinase-free water for a total volume of 25 µl. All reactions were run in triplicate. A standard dilution series with a known amount of DNA containing a single copy of the PCV2 genome was run simultaneously with samples in each reaction (10). The PCR parameters consisted of a 95ºC initial enzyme activation step for 3 min, followed by 38 cycles of a 10 s denaturation at 95ºC, 15 s annealing at 53ºC, and 10 s extension at 72ºC. After amplification, a melt curve analysis was performed to assure the correct product was formed. Quantification of viral genomic copies per ml (GC/ml) of serum and per gram of tissue were then calculated (10).

3.3.10 Sequencing of recovered virus from infected pigs

To confirm that the virus recovered from pigs was the same as that used for inoculation, PCR products from two serum samples in each group were sequenced for both strands with the primers Upper 1 (5' GGTTACACGGATATTGTAGTCCTG 3') and Lower 1 (5' ATTTAATATTGACGACTTTGTTCC 3') at Virginia Bioinformatics Institute using an Automated DNA Sequencer (Applied Biosystems Inc., Foster City, CA). The sequencing results were compared with the sequence of each virus inoculum using MacVector (Accelrys, San Diego, CA).

3.3.11 Statistical analyses

All statistical analyses were performed using the SAS system (version 9.1.3; SAS Institute Inc., Cary, N.C.). The GLM procedure was used for all analyses of variance (ANOVA). Model for ANOVA of rectal temperatures and body weight included treatment (all treatment groups), pig within treatment group as error term for treatment group, day post inoculation (DPI) and the DPI by treatment interaction tested by the overall error term. Logarithmically transformed number of genomic copies in sera, and ELISA results (S/P ratios) of PCV2 antibodies were tested by the same model but with the exclusion of the control group which contained pigs with PCV2 maternal antibodies. Repeatedly measured respiratory scores were dichotomized to either no respiratory distress, or mild to moderate respiratory distress, and then analyzed by logistic regression with treatment group (all 5 treatments), necropsy group and DPI in the model, using the LOGISTIC procedure. Mean number of genomic copies measured in tracheobronchial lymph nodes of inoculated pigs at necropsy at DPI 21 and 42 were compared by ANOVA with treatment group, necropsy group and their interaction in the model. All macroscopic and microscopic lesion scores, as well as immunohistochemistry staining scores in all tissues were analyzed using nonparametric Kruskal-Wallis ANOVA and evaluated based on the Chi-square value using JMPIN (version 4.0.4; SAS Institute Inc., Cary, N.C.). This was followed by Wilcoxon tests if a nonparametric ANOVA test was significant ($P<0.05$), to determine the differences between pairs of groups.

3.4 Results

3.4.1 Construction of mutants and preparation of virus inocula for *in vivo* study

Three PCV2 mutant clones were produced by site-directed mutagenesis: a single mutant R191S with nt 573 mutated from an A to a C; a single mutant P110A with nt 328 mutated from a C to a G; and a double mutant (DM) containing both mutations. DNA sequencing and sequence analysis confirmed these mutations were correctly introduced in the mutants. Upon transfection, all mutant clones were infectious, as determined by IFA with PCV2-specific antibody (9). The infectivity titer for each virus inoculum stock was determined to be $0.5\times10^{5.0}$ $TCID_{50}$/ml for PCV2, $0.5\times10^{4.875}$ $TCID_{50}$/ml for P110A, $0.5\times10^{5.0}$ $TCID_{50}$/ml for R191S, and $0.5\times10^{5.25}$ $TCID_{50}$/ml for DM. The infectivity titers were adjusted, and each pig in each of the groups received the same amount of virus (1.0×10^{5} $TCID_{50}$/ml).

3.4.2 Clinical evaluation of pigs

Over the course of the study, there were no significant differences in pig weights (P=0.94) or rectal temperatures between treatment groups (P=0.30) (data not shown). PCV2-infected pigs were 2.89 (95% CI, 1.80 to 4.62) times more likely than R191S-infected pigs and 1.85 (95% CI, 1.18 to 2.89) times more likely than P110A-infected pigs to have a respiratory score (data not shown). DM-infected pigs were 2.19 (95% CI, 1.37 to 3.52) times more likely to have a respiratory score than R191S-infected pigs.

3.4.3 Gross pathology of lymph nodes and lungs

At 21 DPI, none of the PBS-inoculated (negative control) pigs had enlarged lymph nodes, while at 42 DPI 1/4 pigs had enlarged lymph nodes (Table 3.1). The lymph node enlargement in this pig was not due to PCV2 infection as the pig was not viremic for PCV2 and PCV2 antigen was not detected in the lymph nodes. At 21 DPI, 5/6 PCV2-infected pigs had moderate lymph node enlargement, while 6/6 had moderately to severely enlarged lymph nodes at 42 DPI. At 21 DPI, 3/6 DM-infected pigs had mild lymph node enlargement, while 6/6 had moderate to severe lymph node enlargement at 42 DPI. Three of six P110A-infected pigs had normal to moderately enlarged lymph nodes at 21 DPI, while at 42 DPI 6/6 P110A-infected pigs had moderately enlarged lymph nodes. At 21 and 42 DPI, 5/6 R191S-infected pigs had moderately enlarged lymph nodes. At 42 DPI, pigs infected with PCV2, DM, and P110A had significantly more gross lymph node lesions than the control pigs (P<0.05). There were no significant differences in gross lung lesions between treatments groups

3.4.4 Histopathology of examined tissues

Table 3.2 summarizes the microscopic lesions found in the lung, lymph node, tonsil, spleen, liver, kidney, and small intestine for each group of pigs. The majority of pigs in each group had variable levels of lymphoid depletion and histiocytic replacement in the lymph node, tonsil, and spleen, as well as interstitial pneumonia and lymphohistiocytic inflammation. At 21 DPI, P110A-infected pigs had more severe lymphoid depletion in the lymph nodes, a hallmark lesion of PMWS, than DM-infected pigs (P=0.006) and R191S-infected pigs (P=0.021). The P110A-infected pigs also had more severe histiocytic replacement in the lymph nodes than pigs infected with DM at 21 DPI (P=0.018). By 42 DPI, PCV2-infected pigs had more severe

lymphoid depletion in the lymph nodes than pigs infected with P110A (P=0.007). PCV2-infected pigs also had more significant histiocytic replacement lesions in the lymph nodes than pigs infected with DM (P=0.019), P110A (P=0.011), and R191S (P=0.016). Pigs infected with PCV2 had a higher incidence of small intestine lesions than pigs in all other groups at 42 DPI (P=0.022). Overall, there was a trend of less severe lesions in the DM group than the wild-type group, suggesting attenuation of the DM.

3.4.5 Detection of PCV2-antigen in lymph nodes, tonsil, and spleen

PCV2 antigen was not detected in control pigs at either necropsy (Table 3.3). Three of six PCV2-infected pigs had detectable PCV2 antigen in lymph nodes at 21 DPI, but at this time no pigs had detectable antigen in tonsil or spleen. At 42 DPI, 5/6 pigs infected with PCV2 had antigen in lymph node, 3/6 in tonsil, and 1/6 in spleen. PCV2-infected pigs had significantly higher amounts of antigen detected in lymph node than P110A-infected pigs at 42 DPI (P=0.023). At 21 DPI, 3/6 DM-infected pigs had detectable antigen in lymph nodes, but none in other tissues. At 42 DPI, 4/6 DM-infected pigs had antigen in lymph node, and 1/6 pigs had antigen in both tonsil and spleen. Four of six pigs infected with P110A had PCV2 antigen in lymph node, 2/6 in tonsil, and 1/6 in spleen at 21 DPI. At 42 DPI, 1/6 pigs infected with P110A had PCV2 antigen in lymph node, tonsil, and spleen. At 21 DPI, 2/6 R191S-infected pigs had PCV2 antigen in lymph node and 1/6 had antigen in tonsil and spleen. At 42 DPI, two R191S-infected pigs had antigen in lymph nodes and two in tonsil

3.4.6 PCV2 antibody detection

Due to the presence of PCV2 maternal antibodies in the negative control pigs, the control pigs were removed from statistical analysis of antibody levels. Maternal antibodies waned between 7 and 21 DPI in groups 2-5 (data not shown). There was no difference in PCV2 antibody levels between treatment groups (P=0.40) or over time (P=0.88).

3.4.7 Quantification of PCV2 viremia

The negative control pigs were removed from statistical analysis of viral load data, since, as expected, all pigs were negative for PCV2 DNA for the duration of the experiment. All pigs in groups 2-5 had detectable PCV2 DNA in tracheobronchial lymph nodes (TBLN) during

necropsies (data not shown). There were no differences in the amount of PCV2 DNA detected in the TBLN among any groups (P=0.46). Eleven of twelve PCV2-infected pigs had viremia by 14 DPI, from which time the number of viremic pigs declined (Table 3.4). Eight of twelve DM-infected pigs were viremic at 14 DPI. Eleven of twelve pigs infected with P110A developed viremia by 14 DPI, and thereafter the number of viremic pigs declined. Viremia in P110A-infected pigs appears to have occurred slightly earlier as 7/12 pigs were viremic by 7 DPI compared to 4/12 in PCV2-infected pigs. Nine of twelve R191S-infected pigs were viremic at both 14 and 21 DPI, after which point DNA copies in sera decreased. There were no significant differences in viremia levels between treatment groups (P=0.68) or over time (P=0.59) (Fig. 3.2).

3.4.8 Sequencing of recovered virus

The sequences of the virus recovered from sera of infected pigs were identical to that of the respective inoculum for each group of pigs.

3.5 Discussion

PCV2 associated diseases have become major problems for the global swine industry. The molecular mechanisms of how PCV2 causes PMWS are not well understood, however the PCV2 capsid protein contains main antigenic epitopes and is likely involved in virus virulence (10, 11, 18, 39). We previously reported that two amino acid substitutions in the capsid protein of PCV2 are associated with the attenuation of the virus *in vivo* (11).

To determine if the two amino acid substitutions act singly or collectively in altering PCV2 virulence, we constructed 3 mutant viruses: the R191S and P110A mutants each contained a single amino acid substitution in the capsid protein, while the DM mutant contained both substitutions. *In vivo* virulence study with these mutants showed that, as previously reported (11), there was a general trend towards attenuation in DM infected pigs. As expected, fewer DM-infected pigs developed lesions than wild-type PCV2-infected pigs, and the lesions were less severe than those found in PCV2 infected pigs at both 21 and 42 DPI (Tables 3.1, 3.2) (11). This was particularly apparent with the high level of histiocytic replacement lesions in the lymph nodes (P=0.019), a hallmark lesion of PMWS, at 42 DPI.

Early in the course of infection, P110A-infected pigs had more severe lymphoid depletion and histiocytic replacement lesions in the lymph nodes of infected pigs than in DM-infected pigs. The P110A-infected pigs also developed more lymphoid depletion lesions in lymph nodes than R191S-infected pigs, and more P110A-infected pigs developed viremia by 7 DPI than PCV2-infected pigs. The P110A substitution also occurred during early stage of serial passages of PCV2 in PK-15 cells (within the first 30 passages) (11). Therefore, it is possible that the P110A substitution may affect PCV2 virulence early in the course of infection. By 42 DPI, P110A-infected pigs were less severely affected by infection compared to wild-type PCV2-infected pigs, which was apparent in the severity of the lymphoid depletion and histiocytic replacement in the lymph nodes, small intestine lesions, and the amount of PCV2 antigen detected in lymph nodes of PCV2-infected pigs at 42 DPI.

Importantly, the R191S substitution falls within one of the two conformational neutralizing epitopes in the PCV2 capsid protein identified by Lekcharoensuk et al. (18), further supporting the belief that the R191S substitution is more critical for PCV2 attenuation. However, it appears that both substitutions are necessary for PCV2 attenuation, as there was no clear difference between the infections caused by P110A and R191S at 42 DPI. The combination of these two substitutions may affect virus assembly and alter the structure of the capsid protein, as found with classical swine fever virus (CSFV) (40). Van Gennip et al. (40) found that four amino acid mutations of CSFV resulted in less efficient infection of target cells *in vivo* due to altered affinity for target cells and change in protein surface structure. Similarly, a single amino acid mutation in the hinge region of the envelope protein of Japanese encephalitis virus was found to attenuate the virus by inhibiting viral spread from cell to cell, as well as decrease virus binding to target cells (43). The 3-D crystal structure of PCV2 capsid protein is needed in order to fully understand the effect of the 2 substitutions on capsid assembly and virus binding.

As with PCV2, mutations in the capsid envelope genes of a number of viruses result in virus attenuation. Three amino acid mutations in the E1 glycoprotein of Sindbis virus were found to attenuate the virus (32). When the three mutations were examined individually and collectively, two of the mutations were found to work collectively in virus attenuation, while the third mutation was found to have only a modulatory effect (32). Three amino acid mutations in the virus coding regions were investigated singly and collectively for the attenuation of the Sabin

type 3 oral poliovirus vaccine (41). The amino acid mutation in the structural protein VP3 had a strong direct effect on attenuation, while the other two had no significant effect together or separately on attenuation (41). The VP3 and 472 mutations were analyzed and both were found to be necessary for attenuation of the vaccine strain, while individually they were not fully attenuating (41). Subbarao et al. (36) analyzed three amino acid mutations that resulted in temperature sensitivity and attenuation of influenza A virus, and found that sequential addition of each individual mutation increased attenuation and stability of the virus. Another member of the *Circoviridae* family, chicken anemia virus, was found to have one amino acid mutation in its capsid protein VP1 that rendered it highly attenuated (42).

In summary, the results from this study show that the two amino acid substitutions in the capsid protein of PCV2 are collectively responsible for virus attenuation, although the R191S substitution has greater impact on attenuation.

3.6 Acknowledgements

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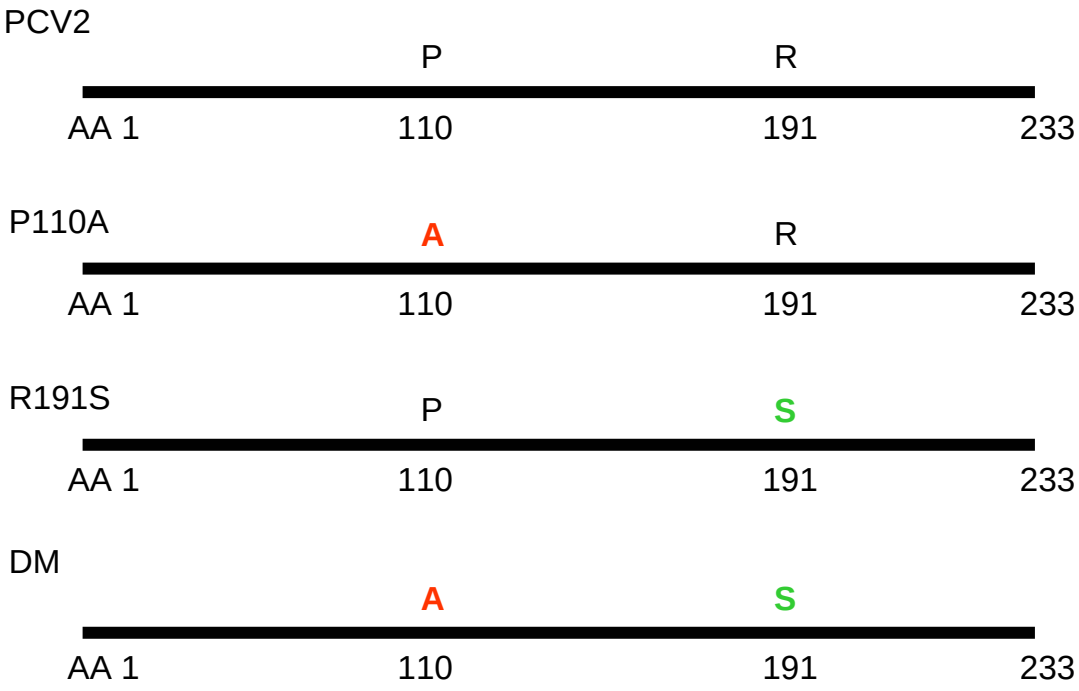

Fig. 3.1. Construction of single mutants P110A and R191S, and double mutant DM was performed by Multi Site-Directed Mutagenesis. Nucleotide 328 of the PCV2 capsid gene was mutated from a C to a G to create a proline to alanine amino acid substitution at position 110 (P110A) of the capsid protein. Nucleotide 573 of the PCV2 capsid gene was mutated from an A to a C to create an arginine to serine amino acid substitution at position 191 (R191S) of the capsid protein. The double mutant (DM) contained both nucleotide mutations and therefore amino acid substitutions.

Fig 3.2.

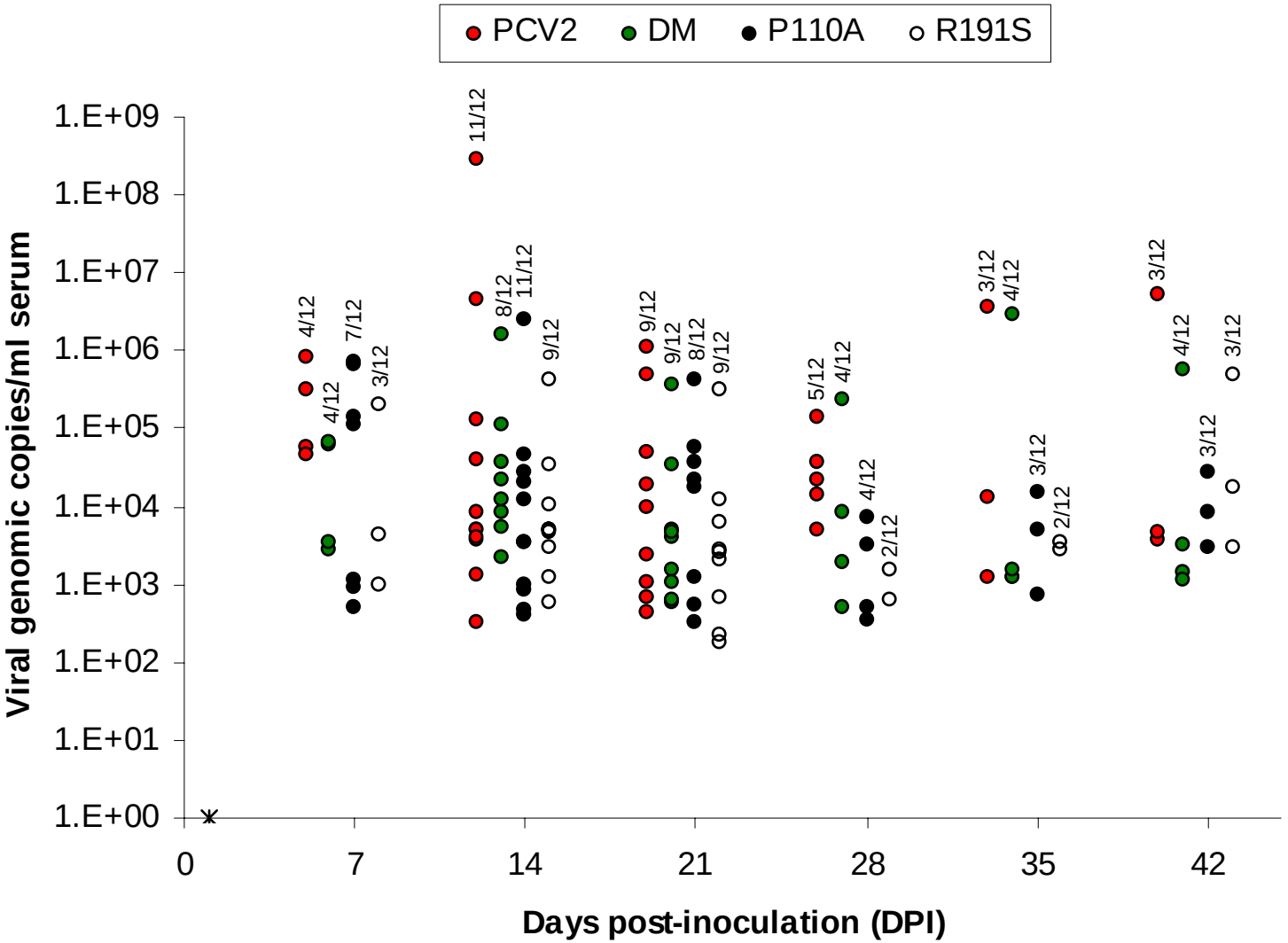
PCV2
DM
P110A
R191S
Viral genomic copies/ml serum
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
0
7
14
21
28
35
42
Days post-inoculation (DPI)
11/12
4/12
7/12
3/12
4/12
8/12
11/12
9/12
9/12
9/12
8/12
9/12
5/12
4/12
4/12
2/12
3/12
4/12
3/12
2/12
3/12
4/12
3/12
3/12

Fig. 3.2. Quantitative PCR results expressed as the log of viral genomic copy numbers in 1 ml of serum (GC/ml) sample collected at -1, 7, 14, 21, 28, 35, and 42 DPI from pigs in groups 2-5. Pigs that were positive for PCV2 DNA are indicated by circles. The number of pigs positive for PCV2 DNA at each DPI are indicated above their respective group (no. positive/total number inoculated).

Table 3.1. Gross lymph node lesions in control and inoculated pigs

Group	Inoculum	No. pigs with enlarged lymph nodes [a]	
		21 DPI	42 DPI
1	PBS buffer	0/4 (0.0)	1/4 (0.3)
2	PCV2	5/6 (1.0)	6/6 (1.8)
3	DM	3/6 (0.7)	6/6 (1.7)
4	P110A	3/6 (0.5)	6/6 (1.2)
5	R191S	5/6 (0.8)	5/6 (1.2)

[a] Four pigs from group 1, and 6 pigs from groups 2, 3, 4, and 5 were necropsied at 21 DPI. The remaining pigs were necropsied at 42 DPI. Values in parentheses are the mean scores of estimated lymph node enlargement (scored from 0=normal to 3=severely enlarged and tan-colored lymph nodes).

Table 3.2. Distribution of histopathologic lesions in different tissues and organs from control and inoculated pigs

Inoculum	DPI	No. of pigs with lesions/no. tested [a]									
		Lung	Lymph Node		Tonsil		Spleen		Liver	Kidney	SI
			LD	HR	LD	HR	LD	HR			
PBS	21	0/4 (0.0)	2/4 (0.5)	0/4 (0.0)	0/4 (0.0)	0/4 (0.0)	0/4 (0.0)	0/4 (0.0)	0/4 (0.0)	1/4 (0.3)	0/4 (0.0)
	42	4/4 (1.0)	2/4 (0.5)	0/4 (0.0)	0/4 (0.0)	0/4 (0.0)	0/4 (0.0)	0/4 (0.0)	1/4 (0.3)	0/4 (0.0)	0/4 (0.0)
PCV2	21	5/6 (1.0)	6/6 (1.5)	5/6 (1.2)	4/6 (0.8)	3/6 (0.5)	4/6 (0.8)	4/6 (0.7)	3/6 (0.5)	1/6 (0.2)	2/6 (0.3)
	42	6/6 (2.0)	6/6 (2.0)	6/6 (1.8)	3/6 (0.8)	2/6 (0.5)	3/6 (0.7)	3/6 (1.0)	3/6 (0.8)	2/6 (0.8)	4/6 (1.0)
DM	21	4/6 (0.8)	5/6 (1.0)	5/6 (0.8)	1/6 (0.2)	1/6 (0.2)	4/6 (0.7)	4/6 (0.7)	5/6 (1.0)	0/6 (0.0)	0/6 (0.0)
	42	4/6 (0.8)	4/6 (1.0)	5/6 (0.8)	1/6 (0.2)	1/6 (0.2)	1/6 (0.2)	2/6 (0.3)	2/6 (0.3)	2/6 (0.3)	0/6 (0.0)
P110A	21	4/6 (0.7)	6/6 (2.0)	6/6 (1.7)	3/6 (0.5)	3/6 (0.5)	3/6 (0.5)	4/6 (0.8)	4/6 (1.0)	1/6 (0.2)	3/6 (0.5)
	42	4/6 (0.8)	5/6 (0.8)	3/6 (0.5)	1/6 (0.2)	0/6 (0.0)	1/6 (0.2)	2/6 (0.3)	2/6 (0.3)	2/6 (0.3)	0/6 (0.0)
R191S	21	3/6 (0.7)	5/6 (1.2)	4/6 (0.8)	3/6 (0.5)	1/6 (0.2)	2/6 (0.3)	4/6 (0.7)	3/6 (0.5)	1/6 (0.2)	1/6 (0.2)
	42	4/6 (0.8)	6/6 (1.3)	4/6 (0.7)	0/6 (0.0)	0/6 (0.0)	1/6 (0.2)	3/6 (0.5)	3/6 (0.5)	0/6 (0.0)	0/6 (0.0)

[a] Values in parentheses are mean histological scores for interstitial pneumonia, lymphoid depletion (LD), and interstitial hepatitis, and histiocytic replacement (HR) for lymph nodes, tonsils, and spleen.

Table 3.3. IHC detection of PCV2 antigen in lymph nodes, tonsils, and spleens of inoculated and control pigs

Group	Inoculum	DPI	No. of pigs with PCV2 antigen/no. tested [a]		
			Lymph node	Tonsil	Spleen
1	PBS buffer	21	0/4 (0.0)	0/4 (0.0)	0/4 (0.0)
		42	0/4 (0.0)	0/4 (0.0)	0/4 (0.0)
2	PCV2	21	3/6 (0.7)	0/6 (0.0)	0/6 (0.0)
		42	5/6 (1.2)	3/6 (0.7)	1/6 (0.2)
3	DM	21	3/6 (0.5)	0/6 (0.0)	0/6 (0.0)
		42	4/6 (0.8)	1/6 (0.3)	1/6 (0.3)
4	P110A	21	4/6 (1.0)	2/6 (0.3)	1/6 (0.2)
		42	1/6 (0.2)	1/6 (0.2)	1/6 (0.2)
5	R191S	21	2/6 (0.5)	1/6 (0.2)	1/6 (0.2)
		42	2/6 (0.3)	2/6 (0.3)	0/6 (0.0)

[a] Values in parentheses are the mean scores of the amount of PCV2 antigen in lymphoid tissues (scores ranged from 0 for no detectable antigen to 3 for high amounts of antigen).

Table 3.4. Detection of virus in sera from inoculated and control pigs by Q-PCR

Group	Inoculum	No. of pigs with viremia/no. tested[a] at DPI:							Total no. of pigs with viremia/no. tested
		-1	7	14	21	28	35	42	
1	PBS buffer	0/8	0/8	0/8	0/8	0/4	0/4	0/4	0/8
2	PCV2	0/12	4/12	11/12	9/12	5/6	3/6	3/6	11/12
3	DM	0/12	4/12	8/12	9/12	4/6	4/6	4/6	11/12
4	P110A	0/12	7/12	11/12	8/12	4/6	3/6	3/6	12/12
5	R191S	0/12	3/12	9/12	9/12	2/6	2/6	3/6	9/12

[a] Four pigs from group 1 and 6 pigs each from groups 2, 3, 4, and 5 were necropsied at 21 DPI. The remaining pigs were necropsied at 42 DPI.

Chapter 4

Construction and *in vitro* characterization of a chimeric porcine circovirus with the PCV1 rep gene in the genomic backbone of PCV2

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Archives of Virology, to be submitted

4.1 Abstract

Porcine circovirus type 1 (PCV1) was originally isolated as a persistent contaminant of the porcine kidney cell line PK-15 and does not cause any disease in swine, whereas porcine circovirus type 2 (PCV2) causes postweaning multisystemic wasting syndrome (PMWS) in pigs. These two viruses have similar genomic organization with 2 main open reading frames (ORF): ORF1 encodes the viral Rep protein involved in virus replication and ORF2 encodes the viral capsid protein. As a persistent contaminant of PK-15 cells, PCV1 has been adapted to grow in PK-15 cells and replicates to titers approximately 1 log higher than that of PCV2. In this study, we created a chimeric PCV with the rep gene of PCV1 replacing that of PCV2 in the genomic backbone of the PCV2. This chimeric virus, SDM-C6, is infectious *in vitro* when transfected into PK-15 cells. The results from a one-step growth curve revealed that SDM-C6 chimeric virus replicates approximately 1 log titer higher than its parental virus PCV2, and has similar titers to PCV1. It has been problematic to grow PCV2 to a higher titer for vaccine production purpose, and even a 1 log titer increase is considered significant. Thus, the results have important implications for PCV vaccine development.

4.2 Introduction

In 1974, porcine circovirus type 1 (PCV1) was originally isolated as a persistent contaminant of the PK-15 cell line ATCC CCL-33 (25). Since its identification, PCV1 has been determined to be a ubiquitous swine virus that does not cause any disease (1, 9, 10, 24). In 1991, a variant strain of PCV, designated as porcine circovirus type 2 (PCV2), was first recognized in pigs in Canada, and is found to be associated with postweaning multisystemic wasting syndrome (PMWS) (2, 22).

Overall, PCV 1 and 2 share 68-76% nucleotide sequence identity in their entire genome, while isolates within each genotype share greater than 90% identity (4). PCV1 and PCV2 have similar genomic organization with two open reading frames (ORF) (6, 7, 18, 23). In both viruses, ORF1 is responsible for viral replication and is alternatively spliced into 2 main functional proteins, Rep and Rep' (6, 7, 17, 18, 20). ORF1 is highly conserved between PCV1 and PCV2 with approximately 83% nucleotide and 86% amino acid sequence identity (22). ORF2 encodes the immunogenic viral capsid protein in both viruses (23), and is more variable than the Rep protein with approximately 67% nucleotide and 65% amino acid sequence identity between PCV1 and PCV2 (22). Recently, a third ORF, ORF3, was identified in PCV2 but not in PCV1, and was reportedly involved in apoptosis (16).

It has been shown that PCV1 replicates better in PK-15 cells and grows to at least a 1-log titer higher than PCV2 (13, 14). This enhanced replication ability of PCV1 in PK-15 cells is likely due to the fact that PCV1 was originally isolated from the PK-15 cell line as a persistent cell culture contaminant, and thus is adapted to grow in PK-15 cells. A problem associated with PCV2 vaccine production is the relatively low titer of PCV2 in PK-15 cells. The unique growth traits of PCV1 and PCV2 make it feasible to construct a chimeric virus with enhanced replication ability. We hypothesize that a chimeric PCV2 virus containing the PCV1 rep gene in the genomic backbone of PCV2 will have enhanced replication ability in PK-15 cells, and thus could serve as a potential strain for future vaccine development.

4.3 Materials and Methods

4.3.1 Cells and DNA clones

PCV-free PK-15 cells were used for the *in vitro* experiments. These cells were previously derived by end-point dilution (11). Constructions of the PCV2 and PCV1 single copy and dimerized tandem repeat infectious DNA clones were previously described (11).

4.3.2 Construction of a chimeric PCV2Gen-1Rep with the ORF1 rep gene of PCV1 replacing that of PCV2 in the backbone of the PCV2 genome

The single copy genome of PCV1 infectious DNA clone in pBluescript II SK+ vector was amplified by PCR with primers PCV1REPF (5' CAACTGGCCAAGCAAGAAAAG 3') and PCV1REPR (5' AACCATTACGATGTGATCAAAAAGACTCAGTAATTTATTTTATATGGGAAAAGGG 3') to produce the PCV1 rep gene fragment with engineered restriction enzyme sites *Bal*I and *Bcl*I at either end. The PCR reaction consisted of 45 µl of Platinum PCR SuperMix High Fidelity (Invitrogen, Carlsbad, CA), 20 pM of primer PCV1REPR, 20 pM of primer PCV1REPF, and 1 µl of the single copy PCV1 infectious DNA clone. The thermocycler reaction consisted of an initial denaturation for 2 min at 94˚C, and 35 cycles of denaturation at 94˚C for 30 s, annealing at 55˚C for 30 s, and extension at 68˚C for 30 s, followed by a final incubation at 68˚C for 7 min. The PCV1Rep fragment was separated by 1% agarose gel, and purified using the GeneClean II Kit (Qbiogene, Irvine, CA). The PCV1Rep fragment was then digested with *Bal*I and *Bcl*I, separately, and the resulting digested fragment was run in a 1% agarose gel and purified using the GeneClean II Kit.

The PCV2 genomic backbone fragment minus the Rep gene was amplified from the single copy PCV2 infectious DNA clone in pBluescript vector by PCR using primers PCV2GENF (5' CTTTTTGATCACTTCGTAATGGTTTTTA 3') and PCV2GENR (5' GCTTACCATGTTGCTGCTGAGGT 3'). The *Bfr*BI and *Bcl*I restriction enzyme sites were introduced at either end of the fragment. The PCR reaction consisted of 20 pM of primer PCV2GENF, 20 pM of primer PCV2GENR, 40 mM dNTP (Fisher Scientific, Pittsburgh, PA), 200mM $MgCl_2$, 10 µl 10X PCR buffer, 72 µl dH2O, 5 units AmpliTaq (Applied Biosystems, Foster City, CA), and 1 µl of the single copy PCV2 infectious DNA clone. The thermocycler reaction consisted of an initial denaturation at 94˚C for 10 min, and 38 cycles of denaturation at 94˚C for 1 min, annealing at 50˚C for 1 min, and extension at 72˚C for 45 s, followed by a final extension at 72˚C for 7 min. The PCV2 genomic backbone fragment (without Rep gene),

PCV2Gen fragment, was separated by 1% agarose gel and purified using the GeneClean II Kit. The PCV2Gen fragment was then digested with *Bfr*BI and *Bcl*I, separately, run on a 1% agarose gel, and purified using the GeneClean II Kit.

To generate the chimeric PCV infectious DNA clone, overnight ligation of the PCV1Rep and PCV2Gen fragments was performed using the Stratagene DNA ligation kit (LaJolla, CA). The ligation mixture was used to transform TOP10 cells (Invitrogen) according to the manufacturer's protocol. White colonies were selected, cultured overnight, and the plasmids were extracted using Sigma's GenElute Plasmid Miniprep Kit (St. Louis, MO). The plasmids were digested with the restriction enzyme *Kpn*I and run on a 1% agarose gel to identify authentic plasmids with 2 bands of approximately 1.7 kb (PCV2Gen-1Rep) and 2.9 kb (pBluescript II SK+ vector).

4.3.3 Viability testing of the chimeric PCV2Gen-1Rep DNA clone by transfection of PK-15 cells

The restriction enzyme *Kpn*I was used to excise the chimeric PCV2Gen-1Rep genome from the pBluescript II SK+ plasmid vector. The chimeric PCV2Gen-1Rep genome was run on a 1% agarose gel, purified using GeneClean II, and subsequently concatomerized with T4 DNA ligase, essentially as previously described (11). PK-15 cells at approximately 70% confluency growing on Lab-Tek chamber slide were transfected with concatomerized PCV2Gen-1Rep genome DNA using Lipofectamine and Plus Reagent according to the manufacturer's protocol (Invitrogen). Three days after transfection, indirect immunofluorescence assay (IFA) using a PCV2 ORF2-specific polyclonal antibody was performed as previously described (11) to determine the infectivity. To further assess the infectivity of the PCV2Gen-1Rep chimeric genome, PK-15 cells at 70% confluency growing in T-25 flasks were transfected with approximately 12 μg of concatomerized chimeric genome per flask as previously described (11). Virus stock was harvested 3 days after transfection and titrated by IFA with a PCV2 ORF2-specific polyclonal antibody as previously described (11).

4.3.4 DNA sequencing to confirm chimeric genome

Primers Rep830F (5' GGTGTCTTCTTCTGCGGTAACG 3') and Rep830R (5' GTTCTACCCTCTTCCAAACCTTCC 3') were used to amplify the junction region between the

3’ of the PCV2Gen fragment and the 5’of the PCV1Rep fragment. Primers Rep10F (5' GGAAGACTGCTGGAGAACAATCC 3') and Rep10R (5' CGTTACTTCACACCCAAACCTG 3') were used to amplify the junction region between the 5’of the PCV1Rep fragment and the 3’of the PCV2Gen fragment. The amplified PCR products were sequenced for both strands.

4.3.5 Site-directed mutagenesis

The initial chimeric PCV2Gen-1Rep DNA clone was not infectious when transfected into PK-15 cells. After analyzing the sequence of the chimeric genome, a 6 nucleotide insertion was identified after the start codon of the PCV1 ORF1 rep gene. To correct this error, primers MVTF (5’ CTCAGCAGCAACATGCCAAGCAAGAAAAGCGG 3’) and MVTR (5’ CCGCTTTTCTTGCTTGGCATGTTGCTGCTGAG 3’) were used to delete the 6 nucleotide insertion using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene). TOP10 cells were transformed with the mutagenized product according to the manufacturer’s protocol (Invitrogen). White colonies were selected and cultured overnight. Clone SDM-C6 was streaked on an LB agar plate containing ampicillin and grown overnight at 37ºC. Four colonies were selected and cultured overnight. Their plasmids were extracted and sequenced using primers Rep830F and Rep830R to ensure that the introduced 6 nucleotides were removed from the chimeric genome.

4.3.6 Preparation of virus stocks for *in vitro* characterization of the chimeric virus

PCV1 and PCV2 virus stocks were prepared, respectively, from PCV1 and PCV2 infectious DNA clones by transfection of PK-15 cells as previously described (Appendix A, 11). The infectious titer of each virus stock was determined by IFA with a PCV2 ORF2-specific antibody (11).

The SDM-C6 chimeric genome containing PCV1 Rep gene in the backbone of PCV2 genome was excised from the pBluescript II SK+ plasmid using the restriction enzyme *Kpn*I, and purified using the GeneClean II kit. Approximately 40 µg of the SDM-C6 chimeric genome was concatomerized with T4 DNA ligase (11) and used to transfect 4 flasks (10 µg per flask) of PK-15 cells at approximately 70% confluency using Lipofectamine and Plus Reagent as previously described (11). Three days post-transfection, the SDM-C6 chimeric virus was harvested by freezing and thawing the transfected cells three times, and the infectious titer of the chimeric

SDM-C6 virus stock was determined by IFA with a PCV2 ORF2 monoclonal antibody (Rural Technologies Inc., Brookings, SD) at a dilution of 1:1000 in Phosphate-Buffered Saline (PBS) (10X, pH 7.4) (Invitrogen) (Appendix A, 11).

4.3.7 One-step growth curve

PK-15 cells were cultured in eight wells of six 12-well plates. At approximately 70% confluency, each well was washed with 2 ml of Minimum Essential Media (MEM) (Invitrogen). Eight wells in duplicate plates were each inoculated with PCV1, PCV2, and SDM-C6 at 0.1 multiplicity of infection (MOI). After1 hour incubation, the inoculum was removed. The cell monolayers were subsequently washed three times each with 2 ml of PBS to remove any excess amount of virus inoculum. Two ml of MEM with 2% fetal bovine serum (FBS) (Invitrogen) and 1X antibiotic-antimycotic (Invitrogen) was added to each well, and the plates were continuously incubated at 37ºC with 5% CO_2. At 0, 12, 24, 36, 48, 60, 72, 84, and 96 hours post-inoculation (hpi), the cells in one well of each duplicate plate were harvested by scraping into the supernatant. The harvested cells were frozen and thawed 3 times and stored at -80ºC until titration. The infectious titer at each hpi was determined in 8-well Lab-Tek II chamber slides (Nalge Nunc International, Rochester, NY) using serially diluted inocula followed by IFA with a PCV2 ORF2 monoclonal antibody using the Spearman-Karber method (11).

4.4 Results

4.4.1 SDM-C6 chimeric genome with PCV1 Rep gene cloned in the backbone of PCV2 genome is infectious *in vitro*

The initial chimeric PCV2Gen-1Rep genome was not infectious after transfection in PK-15 cells. After sequencing of the junction regions of the chimeric genome, a 6 nucleotide (GTAAGC) insertion after the ATG start codon of the PCV1 rep gene, likely introduced during the PCR and cloning steps, was identified in the PCV2Gen-1Rep chimeric genome, which renders the chimeric clone non-infectious.

To correct the unwanted insertion introduced through PCR and cloning steps, site-directed mutagenesis was used to successfully remove the 6 nucleotide insertion. The new chimeric clone, SDM-C6, was found to be infectious upon transfection into PK-15 cells. PK-15 cells transfected with both concatomerized and linearized SDM-C6 genome were strongly

positive by IFA (Fig. 4.1). The SDM-C6 virus stock had a virus infectious titer of 0.5 x $10^{5.5}$ $TCID_{50}$/ml.

4.4.2 The chimeric SDM-C6 virus and PCV1 virus grew to similar titers, which is approximately 1-log higher than that of the parental PCV2 virus

To characterize the growth characteristics of the chimeric virus, and compare it to the wild-type PCV1 and PCV2 viruses, a one-step growth curve was performed. The data showed that the SDM-C6 chimeric virus with a PCV1 Rep gene in the backbone of PCV2 grew as well as the PCV1 virus (Fig. 4.2): both grew to a titer of 2.20 x $10^{4.0}$ $TCID_{50}$/ml at 96 hpi, whereas PCV2 grew to only 2.20 x $10^{3.0}$ $TCID_{50}$/ml at 96 hpi. At12 hpi, the chimeric SDM-C6 virus grew to a titer of 6.95 x $10^{2.0}$ $TCID_{50}$/ml, whereas the PCV1 and PCV2 viruses both had undetectable titers, suggesting that the chimeric virus replicates faster than the parental viruses. PCV1 had a detectable virus titer of 8.70 x $10^{2.0}$ $TCID_{50}$/ml by 24 hpi, whereas PCV2 did not have a detectable titer until 48 hpi (7.91 x $10^{1.0}$ $TCID_{50}$/ml).

4.5 Discussion

PCV1 and PCV2 share similar genomic organization but have distinct pathotype: PCV1 is nonpathogenic, whereas PCV2 causes PMWS, an economically important swine disease. The current licensed and experimental vaccines are all based upon PCV2, such as inactivated whole PCV2 virus, chimeric PCV2 virus or recombinant PCV2 capsid proteins. Unfortunately PCV2 does not grow to a high titer in PK-15 cells, and thus poses a problem for efficient vaccine production. It has been demonstrated that the PK-15 cell culture-adapted PCV1 virus grows better than PCV2, approximately 1-log titer higher than PCV2 in PK-15 cells (13, 14). A 1-log difference may not be significant for other viruses, however for PCV2, a 1-log titer increase could make a major difference in PCV2 vaccine production, as higher titers would reduce the vaccine volume per dose and increase profitability of vaccine manufacturers.

To evaluate if a chimeric PCV2 virus that contains the Rep gene from PCV1 would result in enhanced virus replication in PK-15 cells, we constructed and characterized a chimeric virus, SDM-C6, with the Rep gene of PCV1 replacing that of PCV2 in the backbone of the PCV2 genome including its intergenic sequences. The SDM-C6 chimeric virus is infectious when transfected into PK-15 cells, indicating that the Rep gene between PCV1 and PCV2 are

exchangeable. The growth characteristic of the SDM-C6 chimeric virus was further characterized by a one-step growth curve. It was found that the chimeric SDM-C6 virus replicates more rapidly than their parental PCV1 and PCV2 viruses upon infection of PK-15 cells, and also replicates more efficiently than its parental PCV2 virus with approximately 1-log titer better than PCV2, although the chimeric virus replicates to a similar titer to PCV1.

The mechanism of the enhanced replication ability for the chimeric virus remains to be determined. Since the only difference in the chimeric SDM-C6 virus is a Rep gene from PCV1 (instead of PCV2), it is logical to assume that the Rep gene of PCV1 may contribute to the enhanced replication ability. This hypothesis is further supported by the observation that the chimeric SDM-C6 virus replicates to a similar titer to that of PCV1. We have previously constructed another chimeric virus, designated PCV1-2, with the ORF2 capsid gene of PCV2 cloned into the backbone of PCV1 genome, including its intergenic sequences (12, 14). The PCV1-2 chimeric virus replicated to titers similar to that of PCV2 in PK-15 cells (14). The only difference between the PCV1-2 chimeric virus (12, 14) and the SDM-C6 chimeric virus from this study is the intergenic sequences: the SDM-C6 intergenic sequence is of PCV2 origin, whereas the PCV1-2 chimeric virus is of PCV1 origin. Therefore, it is also possible that the intergenic sequences between the cap and rep genes of the two viruses may play an important role in regulating PCV replication (3, 5, 19).

Upon examining the intergenic sequences, an interferon-α-stimulated response element (ISRE)-like sequence (GAAANNGAAA) was identified in PCV2. The ISRE-element is known to activate gene transcription in response to IFN-α (8). It has been shown that Kaposi's sarcoma-associated herpesvirus expresses viral genes that interact with a viral encoded ISRE-like sequence which is responsible for additional viral gene activation (26). Meerts et al (21) recently showed that both PK-15 and 3D4/31 cells treated with IFN-α after inoculation with PCV2 had an increased number of infected cells, up to 629% and 408%, respectively (21). Interestingly, PK-15 cells treated with IFN-α prior to inoculation with PCV2 had a decreased number of infected cells (69%) (21). In addition to IFN-α, the effect of IFN-γ on PCV2 infection in both PK-15 and 3D4/31 cells have also been evaluated. It was found that treatment with IFN-γ before and after PCV2 infection resulted in a greater number of infected cells, up to 791% and 806% respectively, due to increased cellular internalization of the virus (21). It is possible that a transcription factor responding to IFN-γ activation is present in the promoter region of the PCV2

sequence. Many viruses not only respond to, but also manipulate, IFN expression by a number of transcriptional pathways in order to circumvent cellular IFN response (15). Taken together, the effect of IFN-α and IFN-γ on PCV2 infection in cell cultures and the role of the ISRE-like sequence in regulating IFN-α and IFN-γ responses remain to be studied.

In summary, the chimeric SDM-C6 virus with the Rep gene from PCV1 in the backbone of PCV2 replicates better than its parental PCV2 virus in cell cultures, and thus this chimeric virus could be a candidate for future vaccine development as it contains the immunogenic ORF2 capsid gene of PCV2. It remains to be seen, however, whether or not the enhanced replication ability *in vitro* has any effect on *in vivo* pathogenicity of the chimeric virus.

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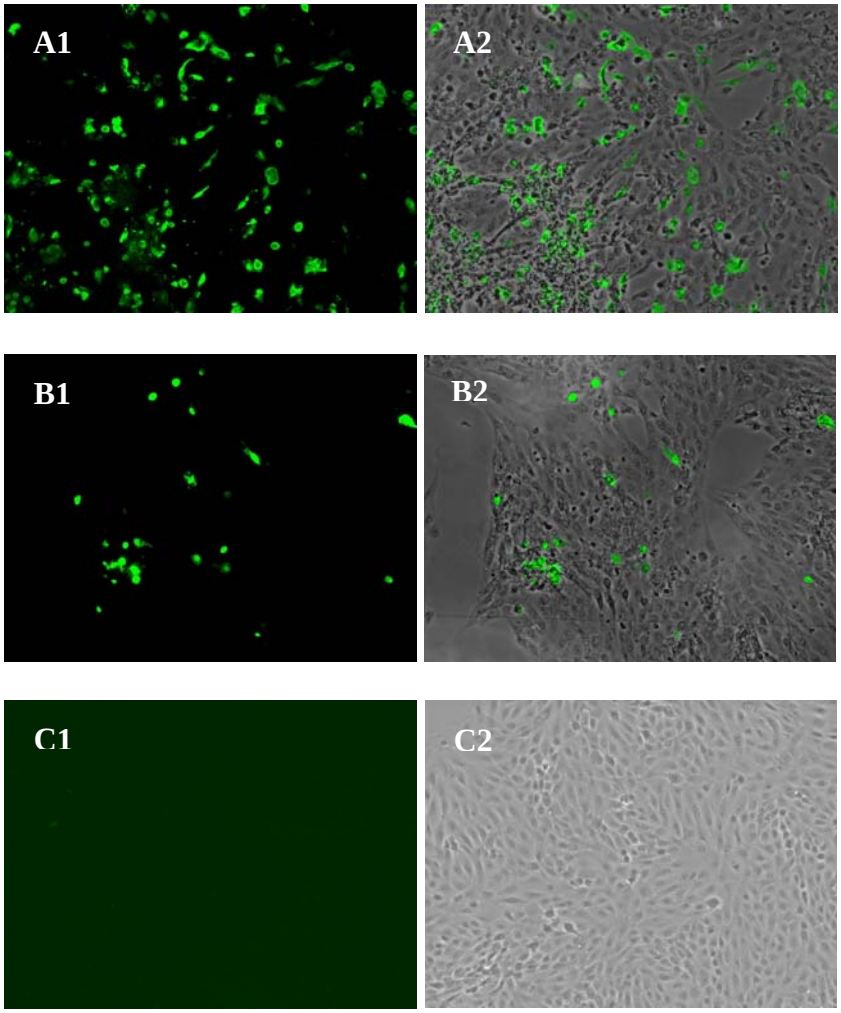

Fig. 4.1. The chimeric SDM-C6 DNA clone (with the Rep gene of PCV1 cloned into the backbone of PCV2 genome) is infectious when transfected into PK-15 cells. **Panels A1 and A2**: PK-15 cells transfected with concatomerized SDM-C6 chimeric genome; **Panels B1 and B2:** PK-15 cells transfected with linearized single copy SDM-C6 chimeric genome; **Panels C1 and C2**: transfection reagents and MEM as negative controls. Left panels: IFA results stained with a PCV2 ORF2 monoclonal antibody; Right panels: PK-15 cell monolayers overlaid with the IFA results.

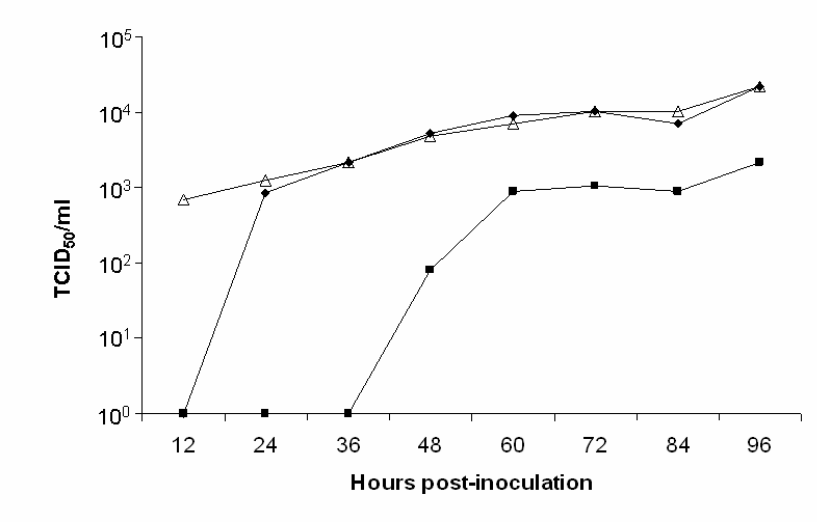

Fig. 4.2. Characterization of the growth characteristics of PCV1 (♦), PCV2 (■), and chimeric SDM-C6 (Δ) viruses in PK-15 cells by one-step growth curve. PK-15 cells on 6- 12 well plates were inoculated in duplicate with each virus at 0.1 multiplicity of infection. Two duplicate wells of infected cells were harvested every 12 hours, and the virus titers were determined by IFA.

Chapter 5

The ORF3 gene of the porcine circovirus type 2 is dispensable for virus replication in pigs

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To be submitted

5.1 Abstract

The open reading frame (ORF) 3 of porcine circovirus type 2 (PCV2) reportedly encodes a novel protein that is involved in apoptosis. To evaluate the effect of ORF3 on PCV2 replication *in vivo*, we created an ORF3-null PCV2 mutant using site-directed mutagenesis. The ORF3-null mutant (muPCV2) was inoculated into pigs: two pigs each were inoculated with lysates of PK-15 cells transfected with dimerized muPCV2 clone at passage 1, two pigs each with lysates of PK-15 cells transfected with dimerized muPCV2 clone at passage 4, and 2 pigs each with 200 μg of muPCV2 dimerized DNA clone. Weekly serum samples were analyzed for PCV2 antibodies by ELISA and muPCV2 viral load by quantitative real-time PCR (Q-PCR). One pig (ID 58) was necropsied at 28 days post-inoculation (DPI), and the remaining pigs were at 35 DPI. All pigs developed viremia starting at 7 DPI, and seroconversion to PCV2 antibodies occurred in all 6 pigs by 21 DPI. muPCV2 DNA was detected from tonsil, mesenteric lymph node (MSLN), lung tissue, and superficial ingunial lymph node of pig 58, and muPCV2 was isolated in PK-15 cells from MSLN. This study demonstrates that the ORF3-null PCV2 mutant DNA clone is infectious *in vivo*, and thus the ORF3 is dispensable for PCV2 replication in pigs.

5.2 Introduction

Porcine circovirus (PCV) was first discovered in 1974 as a contaminant of PK-15 cells (23), and is a member of the virus family *Circoviridae*. The PK-15 cell-derived PCV is non-pathogenic to pigs, and is designated PCV1 (22, 23). A novel strain of PCV, designated PCV2, was isolated in 1998 from piglets with postweaning multisystemic wasting syndrome (PMWS) (1, 15). PCV2 causes multisystemic wasting disease in pigs including such signs as severe progressive weight loss, dyspnea, enlarged lymph nodes, diarrhea, pallor, and jaundice (6, 15). Lymphoid depletion is a hallmark lesion of PMWS and PCV2 infection. It is speculated that apoptosis may be responsible for the observed lymphoid depletion in PCV2 infection.

Shibahara et al (21) reported that PCV2 induces B-lymphocyte depletion via apoptosis. Hirai et al (7) found that one of five piglets inoculated with tissue homogenates from pigs with PMWS developed acute hepatitis, and apoptosis in liver tissue was observed. In mice inoculated with PCV2, apoptosis of infected macrophages were detected in spleen (8). Nuclear lysis and segmentation, and apoptotic bodies were visualized in histiocytes in germinal centers (8). However, several other studies contradicted the role of apoptosis in PCV2 pathogenesis. Mandrioli et al (13) believed that decreased cell proliferation, not apoptosis, was responsible for lymphoid depletion. Dendritic cells harbor infectious PCV2 without any cell modulation, including apoptosis (24). Further comparison of pigs with PMWS and healthy pigs showed that the higher the PCV2 viral load and the severity of PMWS lesions, the lower the apoptosis rate in tissues (20), further contradicting the involvement of apoptosis in PCV2 pathogenesis (9).

Recently, Liu et al (11) reportedly identified a novel protein encoded by ORF3 in the antisense direction of ORF1, and the ORF3 protein expression peaked at 48 hours post-infection in PK-15 cells. Subsequently, Liu et al (11) showed that ORF3 is responsible for the induction of apoptosis in PCV2-infected PK-15 cells. However, the effect of ORF3 on PCV2 replication and pathogenesis in its natural host, pigs, in not known. The objective of this study is to determine, in a pilot animal study, if ORF3 is required for virus replication in pigs.

5.3 Materials and Methods

5.3.1 Virus and cells

PCV-free PK-15 cells were used for the *in vitro* experiments. These cells were previously derived by end-point dilution (3). PCV2 40895 was derived from a pig with naturally

occurring symptoms of PMWS (2). Construction of the PCV2 40895 infectious DNA clone was previously described (3).

5.3.2 Construction of an ORF3-null PCV2 mutant

Site-directed mutagenesis was performed on the PCV2 40895 infectious DNA clone using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) to silence the ORF3 gene. Primers ORF3mufo (5' GGGATGGTTACCACGGTGAAGAAGTGGTTGTTA 3') and ORF3mure (5' TAACAACCACTTCTTCACCGTGGTAACCATCCC 3') were designed to mutate the start codon of the ORF3 gene from ATG to GTG (M to V). The site-directed mutagenesis reaction included 5 µl 10X reaction buffer, 10 ng PCV2 SK+ DNA clone, 12 pM forward primer ORF3mufo, 12.6 pM reverse primer ORF3mure, 1.0 µl dNTP (per manufacturers' instructions), 41.34 µl ddH_2O, and 2.5 units of *PfuUltra* HF DNA polymerase. The reaction was performed in a thermocycler with activation at 95°C for 30 s, then 16 cycles of 95°C denaturation for 30 s, 55°C annealing for 1 min, and 68°C extension for 2 min. The reaction was incubated on ice for 2 min, and subsequently digested with 10 units of *Dpn*I at 37°C for 1 hour. One Shot TOP 10 cells (Invitrogen, Carlsbad, CA) were transformed with the digested reaction according to the manufacturer's protocol.

White colonies were selected and the plasmids were extracted with Sigma's GenElute Plasmid miniprep kit (St. Louis, MO). Two primers, SeqFor (5' GGGTGTGGTAAAAGCAAATGGG 3') and SeqRevC (5' GGGCCAAAAAAGGTACAGTTCC 3'), were used to sequence the region spanning the ORF3 start to verify that the correct mutation was introduced.

5.3.3 *In vitro* viability testing of the mutant

The ORF3-null mutant PCV2 genome was excised from pBluescript II SK+ vector with restriction enzyme *Sac*II, and the genome was concatomerized with T4 DNA ligase. The concatomerized genomes were transfected into PK-15 cells essentially as previously described (3). The transfected cells growing on Lab-Tek chambers were incubated for 3 days at 37°C with 5% CO_2, and then an indirect immunofluorescene assay (IFA) with PCV2- specific ORF2 antibody was performed to assess the viability of the mutant (3).

5.3.4 Construction of a dimerized muPCV2 DNA clone

The muPCV2 genome was excised from the pBluescript II SK+ vector with the *Sac*II restriction enzyme. The digestion product was run on a 1% agarose gel, the 1.7 kb virus genomic DNA band was excised, and purified using the GeneClean II Kit. For dimerization, 4 µg of the *Sac*II linearized muPCV2 genome, 5 µl 10X ligase buffer, 4 units T4 DNA Ligase (Stratagene), and 4 µl dH_2O were incubated at 4°C for 4 hours. The dimerization product was separated on a 1% agarose gel and the 3.4 kb dimerized virus genomic DNA band was excised, and subsequently cloned into the pBluescript II SK+ vector.

To determine if the dimerized clones are infectious, PK-15 cells at approximately 70% confluency growing on a Lab-Tek chamber slide were transfected with the dimerized clones as previously described (Appendix A, 3). Three days after transfection, IFA with a PCV2-specific ORF2 antibody was performed (Appendix A, 3).

5.3.5 Preparation of inocula for *in vivo* study

Three inocula were prepared for the *in vivo* assessment of muPCV2 infectivity: lysates of PK-15 cells directly transfected with 12 µg of muPCV2 infectious DNA clones (passage 1 virus) (3), lysates of PK-15 cells transfected with 12 µg of muPCV2 infectious DNA clones at passage 4 (passage 4 virus), and muPCV2 dimerized plasmid DNA clone.

5.3.6 Design of *in vivo* experiment

Six piglets of three weeks of age were each inoculated with muPCV2 (Table 5.1): 2 piglets with the passage 1 virus (2 ml intranasally and 1 ml intramuscularly), 2 piglets with passage 4 virus (2 ml intranasally and 1 ml intramuscularly), and two piglets each with 200 µg of muPCV2 dimerized DNA clone (3 ml intramuscularly).

Pig number 58 was necropsied at 28 days post-inoculation (DPI), and the remaining pigs were necropsied at 35 DPI. Mesenteric lymph nodes (MSLN), superficial ingunial lymph nodes (SILN), tonsil, and lung were collected from pig 58.

5.3.7 ELISA to detect PCV2 specific antibodies

Blood was collected from each pig 2 days prior to inoculation and weekly thereafter until 35 DPI. ELISA was performed to determine IgG antibodies to ORF2 of PCV2 in sera as

previously described (17) at Iowa State Univeristy Diagnostic Laboratory. A sample/positive (S/P) ratio > 0.2 is considered positive (17).

5.3.8 Quantitative real-time PCR (Q-PCR) to determine viral load

Lung tissue, tonsil, MSLN, and SILN from pig 58 necropsied at 28 DPI and serum from all pigs were tested for PCV2 virus DNA load with a modified Q-PCR (4, 12). Tissues from pig 58 were homogenized at 10% (weight/volume) in cold PBS buffer. Viral DNA was extracted from 200 µl of each lymph node homogenate and serum sample with Qiagen's QIAamp DNA Mini Kit according to the manufacturer's protocol, and eluted in 100 µl of elution solution. The primers (MCV1 and MCV2) used for the quantitative PCR were based on known sequences of PCV2 and PCV1 (2, 4, 5). Each PCR reaction consisted of 7.5 pM of both MCV1 and MCV2 primers, 10 µl of template, 12.5 µl of iQ SYBR Green Supermix, and 1 µl of DNase-RNase-proteinase-free water for a total volume of 25 µl. A standard dilution series with a known amount of DNA containing a single copy of the PCV2 genome was run simultaneously with samples in each reaction (4). All reactions were run in triplicate. The PCR parameters consisted of a 95ºC initial enzyme activation step for 3 min, followed by 38 cycles of a 10 s denaturation at 95ºC, 15 s annealing at 53ºC, and 10 s extension at 72ºC. After amplification, a melt curve analysis was performed to assure the correct product was formed. Quantification of viral loads as genomic copies per ml (GC/ml) of serum and per gram (GC/g) of tissue were calculated as previously described (4).

5.3.9 Sequencing of virus inoculum and virus recovered from pigs

DNA sequencing was performed on the entire genome of each virus inoculum. In addition, the entire genomes of the recovered viruses from pig 58 SILN, MSLN, and lung tissue, and viruses recovered from sera from DPI 14 pigs 61 and 62, and DPI 21 pigs 58, 59, and 62 were also sequenced with PCV2-specific primers for both strains at Virginia Bioinformatics Institute, Blacksburg, VA.

5.3.10 Virus isolation

A 10% tissue homogenate from pig 58 MSLN was used to inoculate PK-15 cells at 70% confluency in a T-25 flask. The flask was washed with MEM, and 1 ml of tissue homogenate

was added. After 1 hour absorption, the inoculum was discarded and 6 ml of fresh medium was added. The flask was incubated for 3 days at 37°C with 5% CO_2, and the virus was harvested by freezing and thawing 3 times. The infectivity of the harvested virus was determined by end-point titration, inoculating serially 10-fold diluted whole lysates onto PK-15 cells grown on Lab-Tek chamber slides followed by IFA with PCV2-specific ORF2 antibodies (Appendix A, 3).

5.4 Results

5.4.1 The ORF3-null PCV2 mutant is infectious *in vitro*

Five ORF3-null mutants were sequenced, and each clone contained the desired introduced point mutation of ATG to GTG but had no other unwanted mutation. The concatomerized viral genome (without vector) was infectious when transfected into PK-15 cells as evidenced by the detection of PCV2 antigen in transfected cells by IFA. Plasmid DNA clones with dimerized genome of the ORF3-null mutant were also transfected into PK-15 cells. IFA staining of the transfected cells with PCV2-specific antibody confirmed that the dimerized DNA clones of the ORF3-null mutant in pBluescript II SK+ vector are infectious.

5.4.2 The ORF3 gene of PCV2 is dispensable for virus replication in pigs

Prior to inoculation, at -2 DPI, serum from all six pigs was negative for PCV2 maternal antibodies (Table 5.2). At 14 DPI, pig 62 seroconverted to PCV2-specific antibodies, while the other 5 pigs seroconverted at 21 DPI. All six pigs developed viremia induced by muPCV2 inoculation (Table 5.3). Viremia was first detected at 7 DPI in pigs 57, 61, and 62. Peak serum viral loads occurred between 14 and 21 DPI, after which time the serum viral load began to decrease. Virus DNA was detected by Q-PCR in MSLN, SILN, tonsil, and lung tissue of pig 58 necropsied at 28 DPI. Viral load in these tissues ranged from 10^9-10^{10} GC/g tissue.

The entire genome of each virus inoculum was sequenced and verified that they contain the desired mutation of the ORF3-null PCV2 mutant clone (Fig. 5.1). The entire genomes of viruses recovered from pig 58 SILN, MSLN, and lung tissue, as well as from sera of DPI 14 pigs 61 and 62, and DPI 21 pigs 58, 59, and 62 were also sequenced and confirmed that they were derived from the original inoculum (Fig. 5.1). Infectious virus was re-isolated on PK-15 cells from lymphoid tissues of pig 58.

5.5 Discussion

PCV2 contains several potential open reading frames, but only two with confirmed functions: ORF1 encodes the virus replication protein Rep, and ORF2 encodes the virus capsid protein Cap (14, 18). Liu et al (11) reported that, in PK-15 cells, the ORF3 protein caused apoptosis and that the mutant PCV2 with a silenced ORF3 had considerably less apoptotic activity. The effect of ORF3 on virus infectivity in pigs is not known.

In this study we successfully created a dimerized infectious DNA clone of ORF3-null PCV2 mutant. Pigs inoculated with three different sources of the mutant viruses all developed active infection as evidenced by seroconversion, viremia, detection of viral DNA in tissues, and virus isolation from tissues of infected pigs. All of the recovered virus sequences were identical to that of the inoculum, indicating that mutating the start codon of ORF3 does not adversely affect virus infectivity *in vivo* or *in vitro*.

Like the ORF3 of PCV2, infectious bursal disease virus's (IBDV) VP5 is not necessary for virus replication but is involved in apoptosis (16, 25). Another circovirus, chicken anemia virus (CAV), encodes apoptin that causes apoptosis in lymphoblastoid T cells and is involved in pathogenesis (19). Using a mouse model, Li et al (10) recently reported that the ORF3-silenced PCV2 apparently is attenuated. It is difficult to interpret the PCV2 pathogenesis data in mice since mice are not the natural host of PCV2. Therefore, it remains to be seen if the ORF3-silenced PCV2 is indeed attenuated in it natural host, pigs, which is beyond the scope of this pilot study.

In summary, we created an ORF3-null PCV2, and determined that the dimerized DNA clone is infectious *in vitro* as well as in pigs. We therefore conclude that ORF3 is not necessary for virus replication in pigs. In the future, the pathogenesis of ORF3-null PCV2 in comparison to the wild-type PCV2 will be assessed in pigs. It is also important to elucidate whether or not ORF3 causes apoptosis in its natural host, pigs.

5.6 Acknowledgements

We would like to acknowledge Jenn Gillespie and Denis Guenette for pig handling help, Pam Mohr for excellent pig care, and Dr. Kijona Key and Jenn DiCristina for assistance with pig handling, euthanasia, and necropsy. Thanks to my committee members Drs. S.M. Boyle, L.A. Eng, W.R. Huckle, S. Tolin, and T. Toth for their support.

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Table 5.1. Experimental design to assess the infectivity of ORF3-null PCV2 mutant

Inocula	Pig ID	Necropsy (DPI)
Passage 1 virus	57	35
	60	35
Passage 4 virus	61	35
	62	35
Plasmid DNA clone	58	28
	59	35

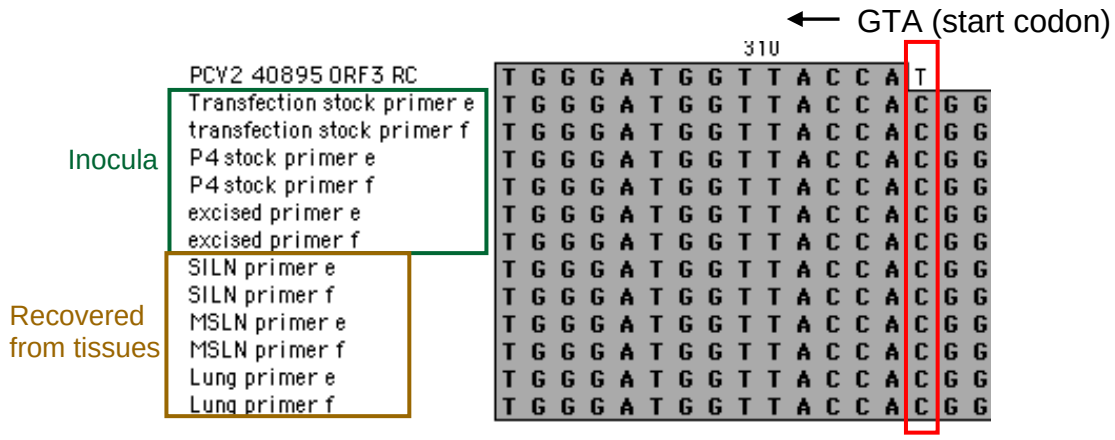
Table 5.2. Seroconversion to PCV2 antibody in pigs inoculated with ORF3-null PCV2 mutant

Inocula	Pig ID	DPI					
		-2	7	14	21	28	35
Passage 1 virus	57	-	-	-	+	+	+
	60	-	-	-	+	+	+
Passage 4 virus	61	-	-	-	+	+	+
	62	-	-	+	+	+	+
Plasmid DNA clone	58	-	-	-	+	+	+
	59	-	-	-	+	+	+

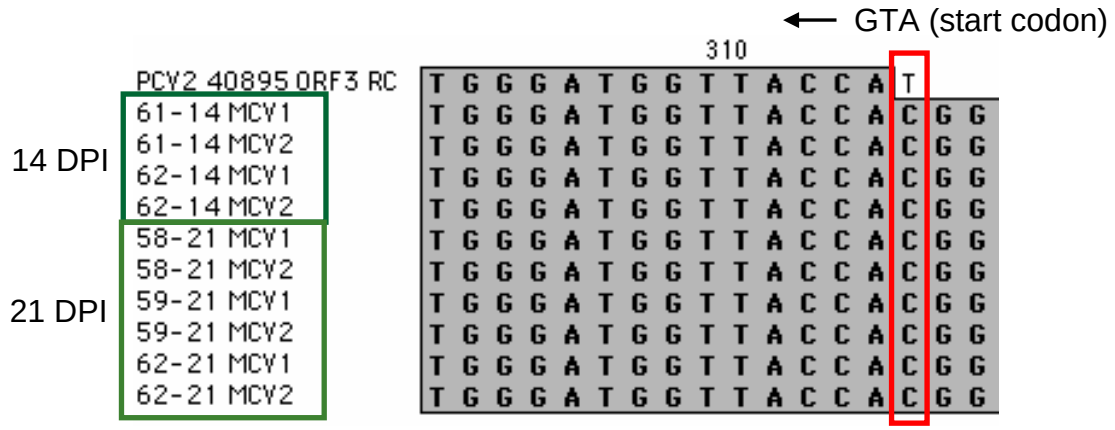
Table 5.3. Serum viral load in inoculated pigs

Inocula	Pig ID	Serum viral load (GC/ml) at DPI:				
		-2	7	14	21	28
Passage 1 virus	57	N/D[a]	6.80E+05	1.50E+06	9.20E+04	3.62E+03
	60	N/D	N/D	2.12E+05	N/D	N/D
Passage 4 virus	61	N/D	5.40E+04	3.00E+07	1.14E+07	8.25E+04
	62	N/D	1.99E+06	2.60E+08	3.76E+07	3.69E+04
Plasmid DNA clone	58	N/D	N/D	N/D	8.75E+08	3.80E+05
	59	N/D	N/D	1.20E+07	3.78E+07	3.71E+05

[a] No virus was detected in this sample.



A.



B.

Fig. 5.1. DNA sequencing was performed on the entire genome of each virus inoculum (Panel A). In addition, the entire genomes of the recovered viruses from pig 58 SILN, MSLN, and lung tissue (Panel A), and viruses recovered from sera from DPI 14 pigs 61 and 62, and DPI 21 pigs 58, 59, and 62 (Panel B) were also sequenced. The entire genome of each virus inoculum and of viruses recovered from tissue and sera were identical, and contained the desired start codon mutation of the ORF3-null PCV2 mutant clone (ATG to GTG).

Chapter 6

In vivo assessment of the role of open reading frame 3 of porcine circovirus type 2 (PCV2) in virus pathogenesis in pigs

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To be submitted

6.1 Abstract

Porcine circovirus type 2 (PCV2) is the primary causative agent of postweaning multisystemic wasting syndrome (PMWS) in pigs. Recently, the open reading frame (ORF) 3 protein of PCV2 was reported to be involved in apoptosis in PK-15 cells and the ORF3-deficient PCV2 was reportedly less pathogenic than PCV2 in BALB/c mice. To definitively assess the role of ORF3 in PCV2 pathogenesis in its natural host, we compared the pathogenicity of an ORF3-null PCV2 mutant (muPCV2) and the wild-type PCV2 in pigs. Thirty-one pigs were divided into 3 groups of 11, 10, and 10 each. In group 1, each of 11 pigs were inoculated intramuscularly (IM) with PBS buffer as negative controls; in group 2, each of 10 pigs were inoculated IM with 200 μg of muPCV2 infectious DNA clone; and in group 3, each of 10 pigs were inoculated IM with 200 μg of PCV2 infectious DNA clone. Blood was collected prior to inoculation and weekly thereafter, and tested for PCV2 antibodies by ELISA and viral DNA loads in the serum by quantitative PCR. All pigs were necropsied at 35 days post-inoculation (DPI) and various tissues were collected and analyzed for gross and microscopic lesions. The results showed that pigs inoculated with the ORF3-null PCV2 mutant had delayed appearance of viremia and seroconversion, decreased viral DNA loads, lower level of S/P ratios of serum IgG antibodies, and lower amounts of PCV2 antigen in mesenteric lymph nodes, thus suggestive of attenuation of the muPCV2. However, there was no significant difference in the average scores of histological or gross lesions in tissues between PCV2-inoculated and muPCV2-inoculated groups. Therefore, data from this study do not fully support the conclusion of an earlier study in mice that ORF3-deficient PCV2 is less pathogenic. This study did show that the ORF3 is

dispensable for PCV2 replication in pigs, but more studies are warranted to fully assess the role of ORF3 in virus attenuation.

6.2 Introduction

Porcine circovirus (PCV) was first discovered in 1974 and is a member of the family *Circoviridae* (20). The original virus, designated porcine circovirus type 1 (PCV1), was isolated from a persistently infected porcine kidney cell line PK-15, and shown to be non-pathogenic to pigs (19, 20). A variant strain of PCV, designated porcine circovirus type 2 (PCV2), was recently isolated from piglets with postweaning multisystemic wasting syndrome (PMWS) in pigs (1, 12). PCV2 is the primary causative agent of PMWS characterized by severe progressive weight loss, dyspnea, lymph node enlargement, diarrhea, pallor, and jaundice in pigs of 7-15 weeks of age (5, 12).

Originally, like PCV1, the genome of pathogenic PCV2, was identified also to contain 2 functional ORFs: ORF1 encodes the Rep proteins responsible for virus replication (11), while ORF2 encodes the immunogenic capsid protein (3, 8, 15). Recently, a 3rd ORF, ORF3, was identified in PCV2 and is presumably translated in the antisense direction in the ORF1 region (10). The ORF3 protein of PCV2 did not have similarities to any known protein (10). Interestingly, the corresponding region of the non-pathogenic PCV1 is considerably longer, 612 bp, and only has 61.5% amino acid identity with ORF3 of PCV2 (10). It has been reported that the ORF3 protein of PCV2 causes apoptosis in PK-15 cells through the activation of caspase 3 and 8 pathways (10). Pathogenesis of an ORF3-deficient mutant PCV2 was evaluated in BALB/c mice (9). The ORF3-deficient mutant was found to be less pathogenic in mice than the wild-type PCV2 (9). However, the pathogenicity of the ORF3-deficient PCV2 in its natural host, pigs, is not known.

To assess the role of ORF3 in PCV2 pathogenesis definitively, we constructed an ORF3-null mutant by mutating the ORF3 start codon from ATG to GTG without changing its coding amino acid in the antisense direction. The pathogenicity of the ORF3-null PCV2 mutant (muPCV2) was compared with that of wild-type PCV2 in pigs. We hypothesize that muPCV2 will be less pathogenic than the wild-type PCV2.

6.3 Materials and Methods

6.3.1 Virus, cells and infectious clones

The construction of PCV2 dimerized infectious clone was previously described (2, 6). PCV-free PK-15 cells were used for the *in vitro* transfection and propagation of viruses. These cells were previously derived by end-point dilution (2).

6.3.2 Construction of an ORF3-null PCV2 mutant

Site-directed mutagenesis was performed on PCV2 infectious DNA clone (isolate 40895) to silence the ORF3 gene using the QuikChange II site-directed mutagenesis kit according to the supplied protocol (Stratagene, La Jolla, CA). Primers ORF3mufo (5' GGGATGGTTACCACGGTGAAGAAGTGGTTGTTA 3') and ORF3mure (5' TAACAACCACTTCTTCACCGTGGTAACCATCCC 3') were designed to mutate the start codon of the ORF3 gene from ATG to GTG (M to V). The resulting ORF3-null mutant (muPCV2) was sequenced for the region spanning the ORF3 start codon to verify that the correct mutation was introduced. The muPCV2 is infectious when transfected into PK-15 cells.

6.3.3 Assessment of the pathogenicity of the muPCV2 in pigs

Thirty-one pigs were divided into 3 groups of 11, 10, and 10. Prior to inoculation, the pigs were tested negative for PCV2 antibodies by ELISA (14) at Iowa State Univeristy Diagnostic Laboratory. Each of 11 pigs in group 1 were inoculated IM with PBS buffer as negative controls, each of 10 pigs in group 2 were inoculated IM with 200 μg of muPCV2 infectious DNA clone (6), and each of 10 pigs in group 3 were inoculated IM with 200 μg of PCV2 infectious DNA clone (2). We have previously demonstrated that pigs inoculated intramuscularly with infectious PCV2 DNA clones initiated active PCV2 infection and developed lesions indistinguishable from pigs inoculated with live PCV2 virus (2). Thus, PCV2 DNA clone can replace live virus for use in pathogenesis studies in pigs (2). Each pig was weighed weekly, and blood was collected from each pig prior to inoculation and weekly thereafter. All pigs were necropsied at 35 days post-inoculation (DPI).

6.3.4 Evaluation of gross pathology

Lungs, tonsil, tracheobronchial lymph nodes (TBLN), mesenteric lymph nodes (MSLN), and superficial inguinal lymph nodes (SILN) of all pigs were evaluated for gross pathology

during necropsy. Lungs were examined *in situ* for gross lesions. The enlargement of lymph nodes and tonsil was determined by measuring for the length, width and total area (cm).

6.3.5 Evaluation of histological lesions

Sections of lung tissue, tonsil, TBLN, MSLN, SILN, and spleen were collected at necropsy and fixed in 10% neutral-buffered formalin. Microscopic lesions were evaluated in a blinded fashion by a board-certified veterinary pathologist (T.L.). Lung tissue lesions were scored for presence and severity of interstitial pneumonia ranging from 0= normal to 6= severe as previously described (4). Tonsil, spleen, and lymph nodes (TBLN, MSLN, SILN) were scored for the presence of lymphoid depletion ranging from 0= normal to 3= severe lymphoid depletion with loss of lymphoid follicle structure and for the presence of inflammation ranging from 0= normal to 3= severe histiocytic-to-granulomatous inflammation with replacement of follicles (17).

6.3.6 Detection of PCV2 antigen by immunohistochemistry

Immunohistochemistry (IHC) was done on formalin-fixed and paraffin-embedded sections of lung tissue, TBLN, SILN, MSLN, spleen, and tonsil for the detection of PCV2-specific antigen as previously described (18). The IHC slides were evaluated and scored in a blinded fashion for the amounts of PCV2 antigen in these tissues. The score ranged from 0= no detectable staining to 3= large numbers of PCV2-antigen diffusely distributed in the tissue section (17).

6.3.7 Detection of PCV2 specific antibodies by ELISA

ELISA was performed as previously described to determine serum IgG antibodies to PCV2 (14) at Iowa State Univeristy Diagnostic Laboratory. A sample/positive (S/P) ratio > 0.2 is considered positive (14).

6.3.8 Quantitative polymerase chain reaction (Q-PCR) to determine serum viral load

All pig sera samples collected prior to inoculation and at 7, 14, 21, 28, and 34 days post-inoculation (DPI) were tested for PCV2 virus DNA load by Q-PCR as previously described (7). Using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA), viral DNA was extracted from 200

µl serum according to the manufacturer's protocol, and eluted in 100 µl of elution solution. Quantification of viral genomic copies per ml (GC/ml) of serum were then calculated as previously described (3).

6.3.9 Sequencing of virus recovered from pigs

Viruses recovered from DPI 28 serum samples collected in the PCV2-inoculated group (pigs 506 and 510) and in the muPCV2-inoculated group (pigs 166, 168, 174, and 175) were amplified for a 766-bp region and sequenced to determine if there are revertants. Primers E (5' CCTGTCTACTGCTGTGAGTACC 3') and F (5' CTCTAGAAATGTGGACCACG 3') amplified a 766 bp fragment spanning the ORF3 start codon region. The PCR products were sequenced for both strands at Virginia Bioinformatics Institute, Blacksburg, VA.

6.3.10 Statistical analysis

Statistical analysis was performed using JMPIN (version 4.0.4; SAS Institute Inc., Cary, N.C.). The continuous data, including pig weights, ELISA S/P ratios, and serum viral DNA load were analyzed by analysis of variance (ANOVA). A Tukey-Kramer HSD test was performed to determine which groups were significantly different, if the p-value from ANOVA was less than 0.05. The non-parametric data, including immunohistochemistry scores, gross lesions scores, and histological scores were analyzed by nonparametric Kruskal-Wallis ANOVA followed by Wilcoxon testing to determine the differences between pairs of groups if the p-value from ANOVA was less than 0.05.

6.4 Results

6.4.1 Clinical evaluation of pigs

There was no significant difference in pig weights between treatment groups (data not shown) for the duration of the study.

6.4.2 Gross pathology of lung, tonsil, and lymph nodes

One pig inoculated with muPCV2 (ID 167) had consolidation in half of the right cranial lung lobe, but other pigs had no visible lung lesions. There were no significant differences in tonsil length ($P = 0.4004$), width ($P = 0.3759$), or total area ($P = 0.6230$) between treatment

groups. No significant differences were found between treatment groups for TBLN and MSLN length ($P = 0.3852$ and $P = 0.1766$, respectively), width ($P = 0.4240$ and $P = 0.9708$, respectively), or total area ($P = 0.6032$ and $P = 0.3885$, respectively). SILN width did not differ significantly ($P = 0.9637$) between treatment groups. However, in PCV2-inoculated pigs, the length and total area of SILN were significantly larger than those of PBS-inoculated pigs ($P = 0.0046$ and $P = 0.0137$, respectively). The total area of SILN in muPCV2-inoculated pigs was significantly larger than PBS-inoculated pigs ($P = 0.0179$), although there was no significant difference in the SILN length between muPCV2- and PBS-inoculated groups ($P = 0.0593$).

6.4.3 Histological lesions

The lesion scores and number of lesions present in the tissues were variable between treatment groups (Table 6.1). Pigs in both PCV2-inoculated and muPCV2-inoculated groups had mild interstitial pneumonia, mild lymphoid depletion in lymph nodes, spleen and tonsil, and mild histiocytic replacement in lymph nodes. No significant differences in lesion scores were found between treatment groups.

6.4.4 PCV2 antigen

Variable levels of PCV2 antigen were detected in lung tissue, TBLN, SILN, MSLN, spleen, and tonsil of muPCV2- and PCV2-inoculated pigs (Table 6.2). There were no significant differences in the amounts of antigen detected in lung tissue ($P = 0.3620$), TBLN ($P = 0.2282$), tonsil ($P = 0.0634$), spleen ($P = 0.1034$), or SILN ($P = 0.1217$) between treatment groups. PCV2-inoculated pigs had significantly more PCV2 antigen detected in MSLN than muPCV2- or PBS-inoculated pigs ($P = 0.0172$ and $P = 0.0012$, respectively).

6.4.5 PCV2 antibodies

All pigs were negative for PCV2 antibodies eight days prior to inoculation (Fig. 6.1). None of the PBS-inoculated control pigs seroconverted to PCV2 over the time course of the study. Beginning at 14 DPI and continuing through the end of the study, there were significant differences in S/P ratios between PCV2 and muPCV2-inoculated pigs ($P < 0.0001$). At 21 DPI, 3 PCV2-inoculated pigs seroconverted to PCV2, and by 28 DPI, all PCV2-inoculated pigs had

seroconverted. Pigs inoculated with muPCV2 did not seroconvert until 34 DPI, at which point 2 of 10 pigs had S/P ratios > 0.2.

6.4.6 Serum viral load

All pigs were negative for PCV2 DNA at -8 DPI (Fig. 6.2). One pig inoculated with PBS (ID 197) had PCV2 DNA detected in serum at 34 DPI (1.14×10^6 GC/ml). Since all PBS-inoculated controls were seronegative throughout the study, this sample is likely due to lab contamination during the PCR process. All other PBS-inoculated pigs were negative for PCV2 DNA for the duration of the study. PCV2-inoculated pigs developed PCV2 viremia beginning at 7 DPI, whereas muPCV2-inoculated pigs had a delayed viremia beginning at 21 DPI (Fig. 6.2). There was a significant difference in serum viral DNA load (GC/ml) between PCV2- and muPCV2-inoculated pigs at 14 DPI ($P = 0.0043$), at 21 DPI ($P = 0.0015$), and at 28 DPI ($P = 0.0018$). Fewer muPCV2-inoculated pigs developed viremia when compared to PCV2-inoculated pigs.

6.4.7 Sequence verification

Viruses recovered from sera of PCV2-inoculated pigs 510 and 506 at DPI 28 contained the intact ORF3 start codon and were identical to their PCV2 plasmid infectious DNA clone inoculum. Viruses recovered from sera of muPCV2-inoculated pigs 174, 168, 166, and 175 at DPI 28 had a silenced ORF3 start codon, and were identical to their muPCV2 plasmid infectious DNA clone inoculum.

6.5 Discussion

PCV2 is the primary causative agent of PMWS, but the molecular mechanism of PCV2 pathogenesis is not known. It is speculated that apoptosis is involved in PCV2 pathogenesis, and the recent identification of a 3rd ORF (ORF3) in PCV2 that reportedly induced apoptosis in PK-15 cells appears to lend some credence to the speculation (10). An ORF3-deficient PCV2 was shown to be less pathogenic in BALB/c mice when compared to wild-type PCV2 (9). However, whether mouse is a valid model for PCV2 diseases, or even for PCV2 infection, remains debatable. Thus, it is important to assess the pathogenicity of the ORF3-deficient PCV2 in its natural host, i.e. pigs.

In this study, we compared the pathogenicity of an ORF3-null mutant PCV2 (muPCV2) and the wild-type PCV2. We found that muPCV2-inoculated pigs had delayed appearance of viremia and delayed seroconversion to IgG PCV2 antibodies compared to wild-type PCV2-inoculated group. Moreover, the ELISA S/P ratios were significantly higher in PCV2-inoculated group ($P < 0.0001$) than in muPCV2 group, all suggestive of virus attenuation of muPCV2. The serum viral DNA loads in muPCV2-inoculated group were also significantly lower at 14, 21, and 28 DPI ($P < 0.005$) than in wild-type PCV2-inoculated group, also an indication of virus attenuation of muPCV2. PCV2-inoculated pigs had significantly more PCV2 antigen detected in mesenteric lymph nodes than muPCV2 inoculated pigs ($P = 0.0172$), and overall the incidence of PCV2-antigen was higher in PCV2-inoculated pigs compared to muPCV2 inoculated pigs. These virological and serological parameters indicated that the muPCV2 is apparently attenuated in pigs. However, the results from gross pathology and histopathology do not fully support the conclusion that the ORF3-deficient PCV2 is less pathogenic, as there were no differences between the PCV2-inoculated and muPCV2-inoculated groups in histological scores or the number of pigs with interstitial pneumonia, lymphoid depletion in lymph nodes, spleen and tonsil, or histiocytic replacement in lymph nodes.

It has been reported that, when the start codon for the 17 kDa nonstructural protein of the infectious bursal disease virus (IBDV) was silenced, the virus was still able to replicate *in vitro* (13) and *in vivo* (21) but had delayed replication *in vivo* and did not cause bursal lesions in chickens (21). The 17 kDa IBDV nonstructural protein was subsequently found to cause apoptosis *in vitro* (22). Another circovirus, chicken anemia virus (CAV), encodes apoptin that causes apoptosis in lymphoblastoid T cells and is involved in pathogenesis (16). However, unlike IBDV and CAV, the reported apoptotic activity of the PCV2 ORF3 (9, 10) has not been independently confirmed, and the reported attenuation of the ORF3-deficient PCV2 in mice is not fully supported by the data obtained in this pig study. This study did show that the ORF3 is dispensable for PCV2 replication in pigs, but more studies are warranted to fully assess the role of ORF3, if any, in virus pathogenesis.

6.6 Acknowledgements

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6.7 References

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Table 6.1. Histological lesions in pigs inoculated with PBS buffer, ORF3-null muPCV2, or PCV2 infectious DNA clone

Inoculum	No. of pigs with lesions/no. tested [a]								
		Tracheobronchial Lymph Node		Mesenteric Lymph Node		Superficial Inguinal Lymph Node		Spleen	Tonsil
	Lung	LD	HR	LD	HR	LD	HR	LD	LD
PBS	3/11 (0.3)	1/11 (0.1)	0/11 (0)	4/11 (0.4)	0/11 (0)	1/11 (0.1)	0/11 (0)	0/11 (0)	0/11 (0)
muPCV2	8/10 (1.3)	9/10 (1.0)	3/10 (0.3)	8/10 (1.0)	5/10 (0.5)	6/9 [b] (0.8)	2/9 [b] (0.2)	2/10 (0.2)	7/10 (0.7)
PCV2	5/10 (0.7)	3/8 [b] (0.6)	2/8 [b] (0.3)	8/10 (1.1)	5/10 (0.5)	6/10 (0.8)	4/10 (0.4)	5/10 (0.5)	5/10 (0.8)

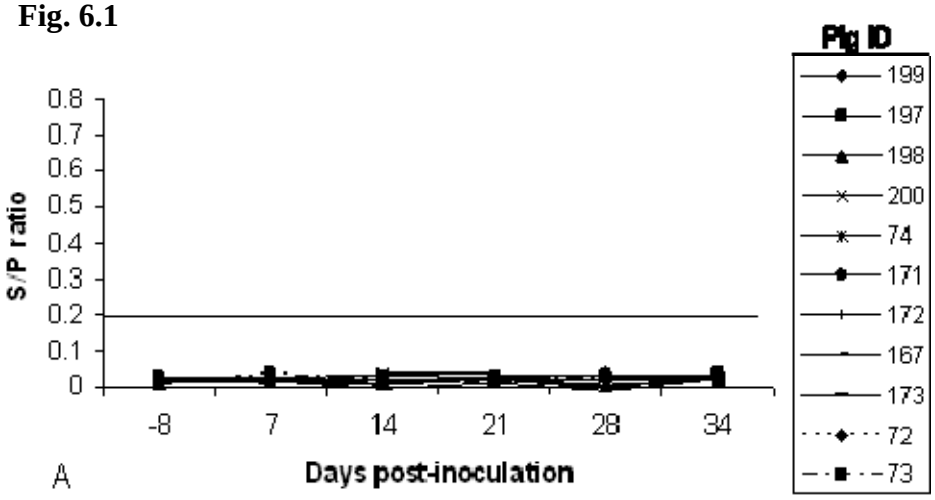
[a] Values in parentheses are mean histological scores for interstitial pneumonia, lymphoid depletion (LD) for lymph nodes, spleen and tonsil, and histiocytic replacement (HR) for lymph nodes.
[b] A few samples were incorrect tissues and were therefore not included in the Table.

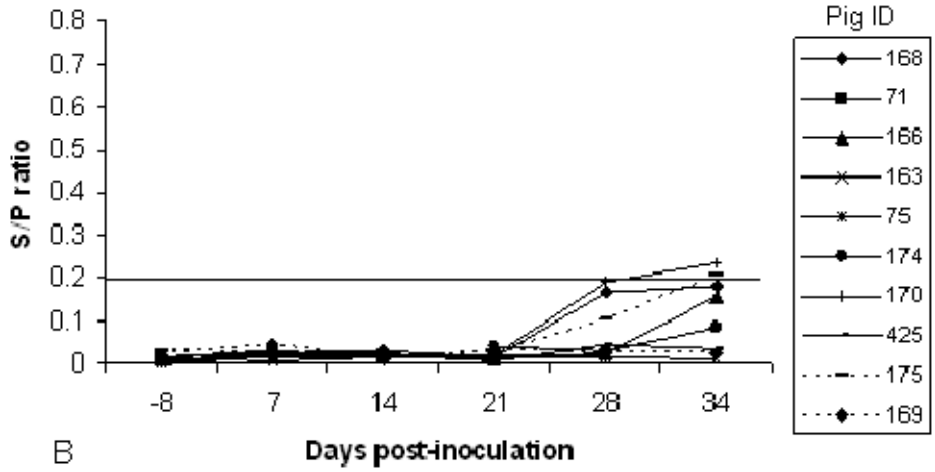
Table 6.2. Detection of PCV2 antigen by PCV2-specific immunohistochemistry in tissues of inoculated and control pigs

Inoculum	No. pigs positive/no. tested (average score):					
	Lung	TBLN	SILN	MSLN	Spleen	Tonsil
PBS	0/11 (0)	0/11 (0)	1/11 (0.2)	0/11 (0)[c]	1/11 (0.1)	1/11 (0.1)
muPCV2	1/10 (0.1)	1/10 (0.1)	1/10 (0.1)	2/10 (0.3)[b]	1/10 (0.1)	3/10 (0.3)
PCV2	0/10 (0)	2/9[a] (0.3)	5/10 (0.6)	7/10 (1.5)[b, c]	4/10 (0.5)	5/10 (0.8)

[a] A tissue sample was missing from the slide.
[b, c] Different superscripts within each column indicate a significant difference in the amount of PCV2 antigen in the tissue between groups.

Fig. 6.1


Pig ID
199
197
198
200
74
171
172
167
173
72
73
S/P ratio
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-8
7
14
21
28
34
Days post-inoculation
A


Pig ID
168
71
166
163
75
174
170
425
175
169
S/P ratio
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-8
7
14
21
28
34
Days post-inoculation
B

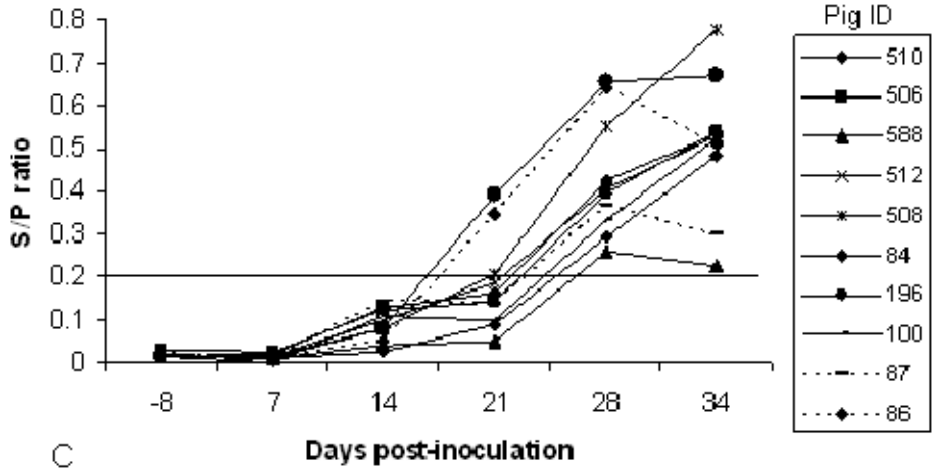

Pig ID
510
506
588
512
508
84
196
100
87
86
S/P ratio
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-8
7
14
21
28
34
Days post-inoculation
C

Fig. 6.1. Seroconversion to IgG PCV2 antibodies measured by PCV2-specific ELISA. A, PBS buffer; B, ORF3-null muPCV2; and C, wild-type PCV2. A S/P ratio equal or greater than 0.2 (indicated by the solid line) is considered positive.

Fig. 6.2

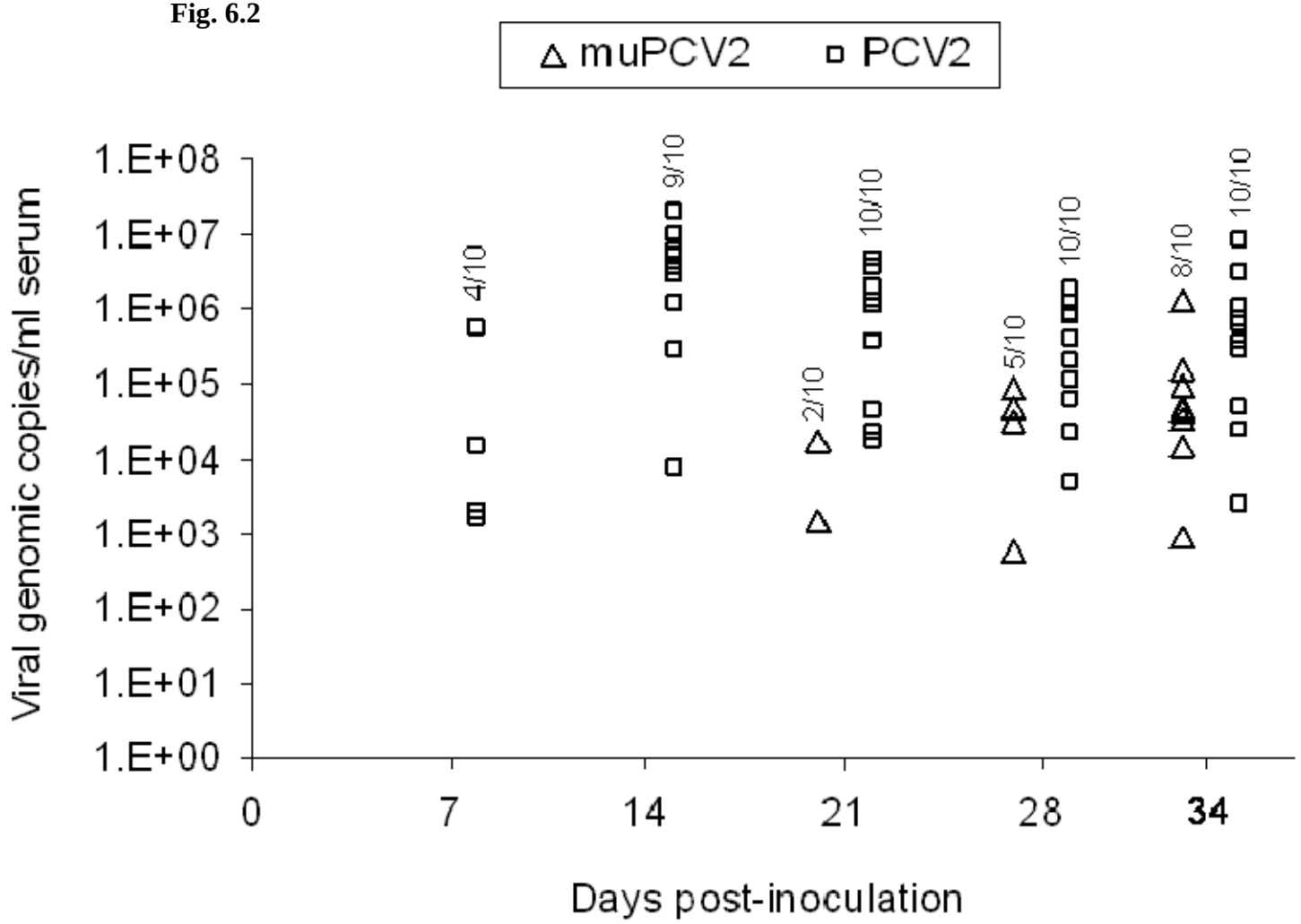

muPCV2
PCV2
Viral genomic copies/ml serum
1.E+08
1.E+07
1.E+06
1.E+05
1.E+04
1.E+03
1.E+02
1.E+01
1.E+00
0
7
14
21
28
34
Days post-inoculation
4/10
9/10
2/10
10/10
5/10
10/10
8/10
10/10

Fig. 6.2. Quantitative PCR results expressed as the log of viral genomic copy numbers in 1 ml of serum (GC/ml) sample collected at 7, 14, 21, 28, and 34 days post-inoculation from pigs inoculated with muPCV2 (Δ) and PCV2 (□) infectious DNA clones. All pigs were negative for PCV2 8 days prior to inoculation. The number of pigs positive for virus DNA at each DPI is indicated above their respective group (no. positive/total no. inoculated).

Chapter 7

General Conclusions and Future Research Directions

Postweaning multisystemic wasting syndrome (PMWS) is an economically important swine disease that is caused by porcine circovirus type 2 (PCV2). A recent report from Smithfield Foods Inc. reported an 8% decrease in the number of hogs it marketed in the latest quarter due to PCV2 infection (www.Reuters.com). In order to stem this loss in the pork industry, it is imperative to understand the mechanisms of PCV2 replication and pathogenesis, and devise effective preventive measures.

The higher the level of PCV2 maternal antibody, the more protection the piglet will have against PCV2 infection, but this does not occur in all pigs. Negative (S/P < 0.2) and low ratios (S/P between 0.2- 0.5) of PCV2 maternal antibodies do not provide protection against PCV2 infection in most cases. An additional study with PCV2 challenge beyond 42 DPI is needed to fully evaluate the effects of prior PCV2 exposure on homologous PCV2 challenge. This experiment would help elucidate if immunity takes longer than 42 days to fully develop. It would be helpful to include another control group of pigs that is inoculated with PCV2 only at 0 days post-inoculation. This could help determine if the pigs were truly infected by the homologous challenge and rule out false-positive results. The role of cell-mediated immunity in PCV2 infection also needs to be investigated in order to clarify the different outcomes of infection despite high maternal antibody ratios. This could be accomplished by performing flow cytometry at different time points to determine specific cell populations in blood; specifically to detect B and T cells.

Previously, 2 amino acid substitutions in the capsid protein of PCV2 that were responsible for increased virus replication *in vitro* and decreased pathogenesis *in vivo* were identified by Fenaux et al (1). It was determined that these two amino acid substitutions, P110A and R191S, act collectively in altering PCV2 virulence in pigs, although the R191S substitution is more responsible for the attenuation. The R191S substitution falls within one of the two conformational neutralizing epitopes in the PCV2 capsid protein identified by Lekcharoensuk et al (2). The 3-D crystal structure of the PCV2 capsid protein is needed in order to fully understand the effect of the 2 substitutions on capsid assembly and virus binding. It would be

beneficial to repeat this experiment with a larger number of pigs, 25 per group, to help elucidate roles of these amino acid substitutions. A bigger sample size would make the results from statistical analysis more reliable.

A chimeric PCV was created with the rep gene of PCV1 replacing that of PCV2 in the genomic backbone of PCV2. This chimeric virus, SDM-C6, was infectious *in vitro* when transfected into PK-15 cells. The results from a one-step growth curve revealed that SDM-C6 chimeric virus replicates approximately 1 log titer higher than its parental virus PCV2, and has similar titers to PCV1. It has been problematic to grow PCV2 to a higher titer for vaccine production purpose, and even a 1 log titer increase is considered significant. Thus, the results have important implications for vaccine development. It would be beneficial to assess SDM-C6 *in vivo* to determine if it elicits a stronger immune response than the parental PCV2 strain, since the virus contains the PCV2 immunogenic capsid protein and has been shown to replicate to higher titers than PCV2 *in vitro*. Also, the intergenic sequences between the Cap and Rep genes of SDM-C6 may play a role in the increased virus replication *in vitro*, as there could potentially be transcription activation sequences in that region. This could be assessed by searching for transcription activation sequences, and subsequently knocking them out by stepwise mutations. The replication of the knockout mutants could then be evaluated by one-step growth curve in comparison to PCV1, PCV2, and SDM-C6.

Recently, the open reading frame (ORF) 3 protein of PCV2 was reported to be involved in apoptosis in PK-15 cells (4) and the ORF3-deficient PCV2 was reportedly less pathogenic than PCV2 in BALB/c mice (3). We created an ORF3-null PCV2 (muPCV2) and determined that the dimerized DNA clone is infectious *in vitro* as well as in pigs; therefore ORF3 is unnecessary for virus replication. The pathogenesis of muPCV2 in comparison to the wild-type PCV2 was assessed in pigs, and it was determined that pigs inoculated with muPCV2 had delayed appearances of viremia and seroconversion, decreased viral DNA loads, lower level of S/P ratios of serum IgG antibodies, and lower amounts of PCV2 antigen in mesenteric lymph nodes, thus suggestive of attenuation of muPCV2. However, there was no significant difference in the average scores of histological or gross lesions in tissues between PCV2-inoculated and muPCV2-inoculated groups. It would be beneficial to repeat this *in vivo* experiment using live virus instead of plasmid, as live virus replicates more rapidly and would quickly provide results, as well as be more representative of natural infection.

It is important to independently verify that the ORF3 of PCV2 is transcribed and translated, as well as causes apoptosis in PK-15 cells. RT-PCR could be done using an antisense ORF3 riboprobe on PK-15 cell lysates harvested at 24-48 hours post-inoculation (hpi) with PCV2 to determine if the ORF3 protein is transcribed. Transcription could also be confirmed by doing a Northern Blot to detect ORF3 RNA. Translation of ORF3 could be assessed by transfecting PK-15 cells with PCV2 and carrying out indirect immunofluorescence assay with an ORF3 specific antibody at 48 hpi. A second way to confirm ORF3 protein expression would be to transfect PK-15 cells with PCV2, harvest lysates at 48 hpi, and use the lysate in Western Blot with an ORF3 specific antibody. Additional protein purification with column chromatography may be necessary. All of these experiments should be carried out simultaneously on muPCV2 to ascertain that ORF3 is silenced.

If transcription and translation of ORF3 are confirmed, experiments to determine if ORF3 causes apoptosis in PK-15 cells could be performed. Lysate from PK-15 cells transfected with PCV2 could be analyzed for poly (ADP-ribose) (PARP-1) activity, which is an enzyme that is activated by DNA breaks and is an inducer of apoptosis, TUNEL could be performed to determine if DNA fragmentation has occurred in transfected cells, or dual staining could be done for antigen and apoptotic cells to determine if PCV2 antigen is present in apoptotic cells. These experiments would be followed by analysis to determine if there is an increase in caspase 3, 8, and 9 activities, which are measures of apoptosis, by any of the many kits that are available through various life science vendors. All of these experiments should be carried out simultaneously on muPCV2 to confirm that ORF3 is silenced.

To determine whether or not ORF3 causes apoptosis in its natural host, pigs, an *in vivo* experiment could be carried out. Three groups of 20 pigs could each be inoculated with PCV2, muPCV2, and PBS (negative controls). Pigs could be necropsied at 14 and 35 days post-inoculation. At necropsy, lymphoid tissues could be collected and immunohistochemistry for PCV2 antigen performed to see if antigen is present in apoptotic lymphocytes and phagocyted apoptotic bodies, and TUNEL could be done to determine if DNA strand breaks in lymphoid tissue cells occurred. Electron microscopy could reveal chromatin condensation in the nuclei of apoptotic lymphocytes along with viral particles. Nuclear lysis and segmentation and apoptotic bodies could be visualized in histiocytes in germinal centers by transmission EM.

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Chapter 8

Molecular characterization of porcine TT virus, an orphan virus, in pigs from 6 different countries

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*This chapter is part of my PhD dissertation. It was originally my MS project, but was never used and later directly transferred to my PhD project.

8.1 Abstract

Human TT virus (TTV), originally isolated from a patient with post-transfusion hepatitis in 1997, is ubiquitous and non-pathogenic. Viruses related to human TTV have since been identified in non-human primates, bovine, ovine, porcine, feline, and canine. The objective of this study was to genetically characterize porcine TTV from pigs in different geographic regions. PCR primers based on the non-coding region of the only available porcine TTV isolate were designed to amplify porcine TTV DNA from sera of pigs in 6 different countries. Porcine TTV DNA was detected in 66.2% (102/154) of the swine sera. The percentages of positive pigs varied greatly from country to country and even within the same country: 33% in Iowa, USA, 40% in Thailand, 46% in Ontario, Canada, 80% in China, 85% in Korea, 90% in Spain, 100% in Quebec and Saskatchewan, Canada. A total of 40 porcine TTV isolates (5 from each geographic region) were sequenced for a 218 bp fragment within the non-coding region. Sequence analyses revealed that porcine TTV isolates from different geographic regions shared 86-100% nucleotide sequence identity to each other. The prototype Japanese isolate of porcine TTV, Sd-TTV31, shared 90-97% nucleotide sequence identity with porcine TTV isolates reported in this study. Phylogenetic analysis showed that the clustering of the porcine TTV isolates is not associated with geographic origins. Although porcine TTV is not known to be associated with any swine disease, coinfection of pigs with TTV and other known swine pathogens may result in enhanced

disease. There are also concerns for risk of potential human infection during xenotransplantation.

8.2 Introduction

TT virus (TTV) was originally isolated from a Japanese patient with post-transfusion hepatitis in 1997 and was named after the initials of the index patient (9). Approximately 92% of healthy Japanese individuals tested positive for TTV DNA, and the role of TTV in liver disease is not known (13). TTV was initially thought to be a member of the *Circoviridae* family along with porcine circoviruses 1 and 2 (PCV), psittacine beak and feather disease virus, chicken anemia virus (CAV) and TTV-like mini-virus (TLMV) (8, 11, 14, 15). Recent studies placed TTV in the newly proposed family *Circinoviridae* (1, 6, 8, 14). TTV is a non-enveloped, single-strand, circular DNA virus of about 3.8 kb and has a diameter of 30-32 nm (2). The viral genome consists of four open reading frames (ORF) that are highly divergent, and a relatively conserved non-coding region of approximately 1.2 kb with 83.4% nucleotide sequence identity among human isolates (11). TTV-like viral sequences have now been detected from swine, bovine, chicken, ovine, and various nonhuman primates (4, 10). The genomic length of TTV isolates appears to be smaller as the order of the animal decreases (12).

Porcine TTV has similar genomic organization with human TTV, but shares less than 45% nucleotide sequence identity. Compared to human TTV, the genome of porcine TTV is smaller, 2.9 kb in length, and contains 3 ORFs (12). By using primers derived from human TTV, Leary et al. (4) found that 4 of the 20 swine tested were positive for TTV DNA based on a 243 bp segment. So far only one strain of porcine TTV has been fully genetically characterized (12). The genetic relationship among porcine TTV isolates in different geographic regions has yet to be determined. In this study, we detected and characterized TTV isolates among swine from six different countries. A 218 bp sequence within the non-coding region of 40 porcine TTV isolates in different geographic regions were determined and analyzed.

8.3 Materials and Methods

A total of 154 convenience swine serum samples were collected from pigs of different geographic regions in six different countries. The health condition of these pigs was not known. Twenty sera were taken from pigs in 4 different commercial swine farms near Beijing, China, 20 sera from pigs in four commercial swine farms in Thailand, 20 sera from pigs of various ages from commercial farms in Korea, 38 sera from pigs of various ages in several commercial swine herds in three geographic regions (Quebec, Saskatchewan, and Ontario) of Canada, 36 sera from

pigs of various ages in Iowa, USA, and 20 sera from pigs in Spain. The sera were stored at an -80°C freezer until used. The sources for some of the serum samples were described previously (Meng et al., 1999).

Viral DNA was extracted from the serum samples using the QIAamp DNA MiniKit according to the protocol supplied by the manufacturer (Qiagen, Inc.). PCR primers were designed based on the first 318 bp sequence within the noncoding region of the only available porcine TTV isolate, Sd-TTV31 (12). Primer pair Upper 1 (5'-TACACTTCCGGGTTCAGGAGGCT- 3') and Lower 1 (5'-GAGTGCAGTTCCGAATGGCTGAGT- 3') was used for the first round PCR to amplify a 271 bp sequence within the non-coding region of the viral genome. The PCR reaction consisted of 1 µl of each 10 µM primer, 2 µl of 10 mM dNTP, 4 µl of 25 mM $MgCl_2$, 5 µl of 10 X PCR buffer, 0.5 µl of AmpliTaq Gold polymerase (Applied Biosystems), 1 µl of extracted DNA, and 36.5 µl of DNase, RNase, and protease free water for a total reaction volume of 50 µl. The PCR parameters included 40 cycles of denaturation at 94°C for 1 min., annealing at 52°C for 1 min., and extension at 72°C for 30 sec., followed by a final incubation at 72°C for 7 min. Primer pair Upper Nested (5'-CAATTTGGCTCGCTTCGCTCGC- 3') and Lower Nested (5'-CGTTCCCACTAAAGTGAATATAAGTGAGTGCAGT- 3') was used in the nested PCR. The expected product in the second round nested PCR is 218 bp.

The PCR product with expected size from the nested PCR was excised from a 0.8% agarose gel. PCR products from 5 selected porcine TTV isolates in each geographic location were purified using the glassmilk procedure with a GENECLEAN III kit (Bio 101 Inc.). The purified PCR products were sequenced for both strands at the Virginia Bioinformatics Institute using an Automated DNA Sequencer (Applied Biosystems Inc.). The sequences were analyzed with MacVector and AssemblyLign (Oxford Molecular Inc.) computer programs. The percentages of nucleotide sequence identities among the different porcine TTV isolates were determined using MacVector. Phylogenetic analysis was performed with the aid of the PAUP program (David L. Swofford, Smithsonian Institute, Washington, DC, distributed by Sinauer Associates Inc., Sunderland, MA).

8.4 Results

A total of 154 swine serum samples collected from pigs in 6 different countries were tested by PCR for the presence of porcine TTV DNA. Overall, 66.2% of the pigs were positive for TTV DNA (Table 8.1). Based on the limited samples tested in each country, the detection rates varied from country to country, and even within the same country: 33.3% from pigs in Iowa, USA, 80% from pigs in Beijing, China, 40% from pigs in Thailand, 85% from pigs in Korea, 100% from pigs in Saskatchewan and Quebec, Canada, 46.2% from pigs in Ontario, Canada, and 90% from pigs in Spain.

Sequence analysis showed that porcine TTV isolates from pigs in different geographic regions shared 86-100% nucleotide sequence identity to each other. The percentages of sequence identity in the non-coding region among different isolates are apparently not associated with the origins of the pigs: 89-96% identity among Thailand isolates, 93-97% identity among Spanish isolates, 91-100% identity among isolates from Saskatchewan, 88-100% identity among Quebec isolates, 93-97% identity among isolates from Ontario, 89-96% identity among Korean isolates, 88-95% identity among Iowa isolates, and 92-97% identity among the Chinese isolates. The prototype Japanese isolate, Sd-TTV31, shared 90-97% nucleotide sequence identity with porcine TTV isolates reported in this study.

Phylogenetic analysis revealed that there is no distinct pattern of clustering of these isolates that are related to geographic origins (Fig. 8.1). No single branch consists of isolates from the same geographic region.

8.5 Discussion

Porcine TTV is an orphan virus in pigs that is not known to be associated with any swine disease. Porcine TTV is genetically related to human TTV and shares similar genomic organization. Thus far only one strain of porcine TTV has been fully characterized (12). Leary et al. (4) tested a small number of pigs and showed that 4 of the 20 pigs tested were positive for TTV DNA. However, the extent of genetic variation among porcine TTV isolates from different geographic regions is not known. We report here the detection and characterization of porcine TTV from pigs in different geographic regions of the world.

In this study, based on the limited samples tested, we found that approximately 66% of the pigs in 6 different countries are positive for porcine TTV DNA, suggesting that, like human TTV, porcine TTV is ubiquitous in swine populations. The percentage of positive pigs varied

greatly from country to country and even within the same country. The mechanism of porcine TTV transmission among pigs is not known. The ubiquitous nature of porcine TTV in swine herds and the lack of apparent geographic clustering of these isolates suggest that common sources for transmission may exist. It is possible that the production of vaccines in swine or contaminated swine cell cultures could lead to worldwide spread and transmission of the virus. It is also possible that TTV is readily transmitted from infected to naive pigs through the administration of vaccines in herds where needles are not changed between injections, which is common in the swine industry. Porcine TTV isolates from pigs in different geographic regions vary from 86-100% nucleotide sequence identity, whereas human TTV isolates diverged from each other as much as 30% (7). Contamination of vaccines, transmission of TTV from pig-to-pig through vaccination procedures, and the common practice of moving large numbers of pigs from site-to-site as they progress through different growth stages in the current swine industry could explain the relatively high nucleotide sequence identity among porcine TTV isolates in different geographical regions. Studies are warranted to determine if swine cells used for vaccine productions and porcine derived commercial products such as pepsin are contaminated by the porcine TTV.

A potential concern regarding porcine TTV is the risk of human infection during xenotransplantation with pig organs and cells. Although porcine TTV is non-pathogenic in pigs, it might become pathogenic due to species jumping in immunosuppressed xenotransplant recipients. Therefore, it is important to screen xenograft donor pigs for the presence of porcine TTV. The role of porcine TTV in the disease manifestation during co-infection with another swine pathogen is also unknown. Recent studies on PCV2 showed that pigs co-infected with PCV2 and porcine parvovirus developed more severe clinical diseases (3).

8.6 Acknowledgements

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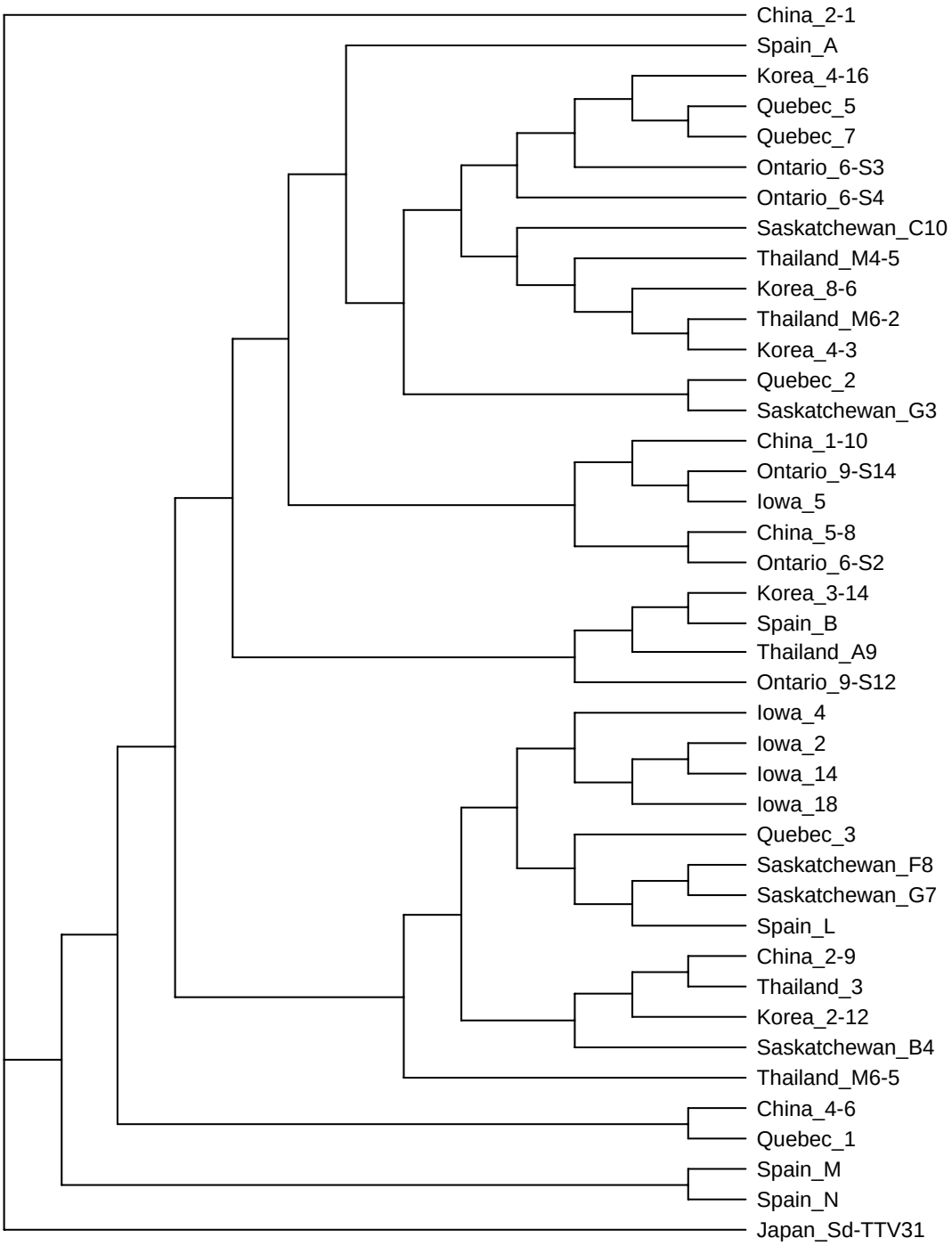
Fig. 8.1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Saskatchewan_C10
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Korea_8-6
Thailand_M6-2
Korea_4-3
Quebec_2
Saskatchewan_G3
China_1-10
Ontario_9-S14
Iowa_5
China_5-8
Ontario_6-S2
Korea_3-14
Spain_B
Thailand_A9
Ontario_9-S12
Iowa_4
Iowa_2
Iowa_14
Iowa_18
Quebec_3
Saskatchewan_F8
Saskatchewan_G7
Spain_L
China_2-9
Thailand_3
Korea_2-12
Saskatchewan_B4
Thailand_M6-5
China_4-6
Quebec_1
Spain_M
Spain_N
Japan_Sd-TTV31

Fig. 8.1. A phylogenetic tree of porcine TT virus isolates from pigs in different geographic regions. The tree was constructed using the parsimony neighbor joining method with the aid of the PAUP program. Bootstrap values are indicated above major branches. The Japanese prototype porcine TTV isolate, Sd-TTV31, was included in the analysis.

Table 8.1. Detection of porcine TT virus DNA by PCR in swine serum samples from different geographic regions

Geographic region	No. of swine sera tested	No. of swine sera positive	% of swine sera positive
Iowa, USA	36	12	33.3
Bejing, China	20	16	80.0
Thailand	20	8	40.0
Korea	20	17	85.0
Saskatchewan, Canada	15	15	100.0
Ontario, Canada	13	6	46.2
Quebec, Canada	10	10	100.0
Spain	20	18	90.0
Total	**154**	**102**	**66.2**

Appendix A

Experimental Protocols

Transfection of PK-15 cells in T-25 flasks

-want cells 70-80% confluent

-use 12µg DNA/flask

EX. 12µg DNA / 120µg/ml = 12µg DNA / 0.12µg/µl = 100µl DNA/flask

1. X µl DNA * x flasks = total amount of DNA
   X µl f-MEM * x flasks = total amount of f-MEM (nothing in MEM)
   350µl total

   -add 16µl Plus Reagent * x flasks = x µl Plus Reagent
   -mix with above and keep at room temperature (RT) for 15 minutes

2. 350µl DNA * x flasks = total amount of f-MEM
   50µl Lipofectamine * x flasks = total amount of Lipofectamine
   400µl total
   -mix

3. Mix 1 and 2 and keep at RT, 15 min.

4. Wash cells in T25 flasks with 5ml f-MEM

   -pour off

   -add 765µl 'reaction 3'/flask

5. 3 hours at 37°C, 5% CO_2 (rock flask each hour)

6. Add 8ml m-MEM (2% FBS, 2X antibiotic-antimycotic)

7. Freeze/thaw cells 3 times at 3 days (72 hours) post-transfection (-80°C)

   Invitrogen:

   -Plus Reagent: Cat. # 11514-015

   -Lipofectamine Reagent: Cat. # 18324-012

IFA with PCV2 monoclonal antibody

1. Pour media out of culture flask (ex. 8 well Lab Tek chamber slides)
2. Wash cells with PBS one time- gently
3. Fix with cold 80% acetone and 20% methanol- 4 drops/well

   Incubate at 4ºC for 20 minutes

   Pour off and wash with PBS one time- gently
4. Dilute PCV2 monoclonal antibody 1:1000 in PBS
5. Add 100 µl/well; Incubate 1 hour at 37ºC in 5% CO_2
6. Pour off and wash 3 times with PBS- gently
7. Dilute FITC-anti-mouse 1:50 in PBS
8. Add 100 µl/well; Incubate 45 minutes at 37ºC in 5% CO_2
9. Pour off and wash 1 time with PBS- gently
10. Dry and add a few drops of Fluoromount-G to slide and put on a coverslip
11. View under fluorescent microscope

Nicole McKeown Juhan was born on April 16, 1979, in Pittsburgh, PA. After finishing high school in Glenshaw, PA, in May 1997, she attended Virginia Polytechnic Institute and State University, Blacksburg, VA, where she was graduated with Bachelors of Science degrees in Biology and Business Management, in May 2002. In May of 2002, she enrolled in a Doctor of Philosophy program at the VA-MD Regional College of Veterinary Medicine, Department of Biomedical and Veterinary Sciences. She continued her academic pursuit under Dr. X.J. Meng's supervision. She is the daughter of Dorothy and Len Ward and Dennis and Kathy McKeown. She is married to Michael Juhan, and has 2 birds and a puppy.